Molecular mechanisms of neuronal dense core vesicle release

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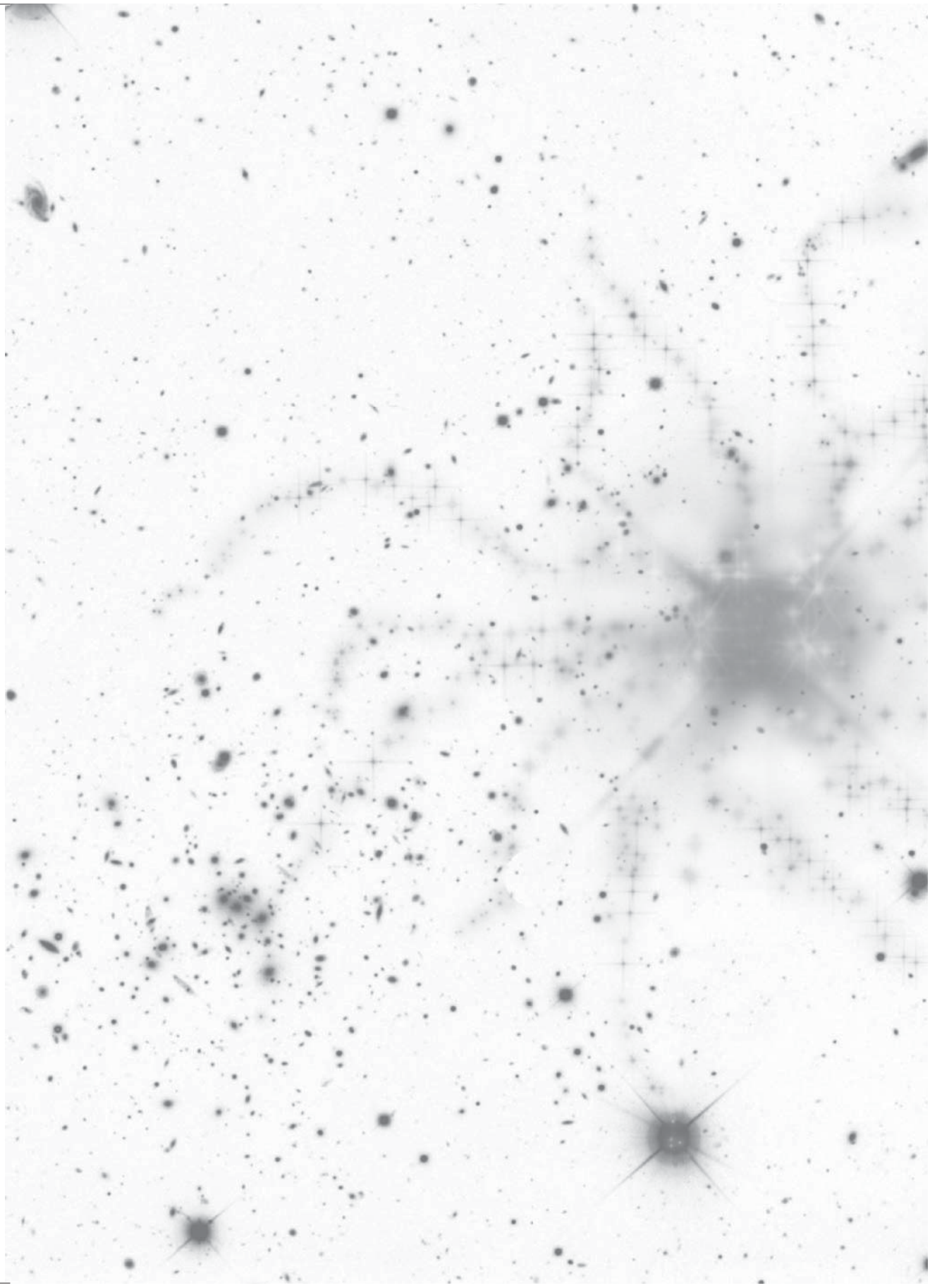


CHAPTER 1

INTRODUCTION

1

ABSTRACT

Regulated secretion of chemical signals in the brain occurs mainly from two different organelles, synaptic vesicles (SVs) and dense core vesicles (DCVs). SV release of neurotransmitters is well described and the proteins that comprise the release machinery for the local and fast release of SVs have been identified. In contrast to our detailed knowledge on SV secretion, the mechanisms that drive DCV release of neuro-modulatory cargo, including many neuropeptides, are less clear and several fundamental questions remain. The main aim of this thesis is to provide detailed mechanistic insight in neuronal DCV release principles. This introduction describes general vesicular release mechanisms in the nervous system and discusses DCV transport and release characteristics in model systems other than neurons. Finally it describes proteins involved in SV secretion and discusses their potential role in DCV release.

1 VESICULAR TRANSPORT IN THE NERVOUS SYSTEM

Neurons are highly polarized cells, with an axon extending towards target cells and a dendritic tree organized to receive incoming signals. Neurons relay most of their signals via chemical synapses, which can be located at significant distances from the cell soma. To supply these synapses, many proteins, lipids and other molecules are actively transported through the cell. The majority of intracellular transport in neurons occurs via vesicular trafficking along microtubule tracks. Different classes of motor proteins are attached to vesicles and regulate their transport (Harada et al., 1998; Hirokawa, 1998; Hirokawa et al., 1990; Schlager and Hoogenraad, 2009; Vallee et al., 1988; Vallee et al., 2004). Neurons harbor several types of vesicles: synaptic vesicles (SVs), synaptic vesicle precursors (SVPs), Piccolo-Bassoon transport vesicles (PTVs), recycling endosomes (RE) and dense core vesicles (DCVs) (see Figure 1). SVs contain classical neurotransmitters and SVPs transport synaptic proteins mainly during synaptogenesis. Neuropeptides and guidance molecules are transported inside DCVs, and most active zone proteins are transported in Piccolo-Bassoon vesicles during early development. In addition, neurons also transport other organelles like mitochondria and lysosomes. Some of these vesicles are secreted in an activity-dependent manner. In this introduction we will discuss the release of SVs and DCVs in depth.

2 VESICULAR RELEASE IN THE NERVOUS SYSTEM

In the brain, neurons communicate mainly via the release of secretory vesicles. An action potential triggered at the cell soma/axon hillock arrives at the synaptic terminal and depolarizes the membrane, which results in calcium influx. In a highly regulated manner, calcium triggers the release of SVs containing neurotransmitter from a specialized region at the membrane called the active zone. After fusion of the vesicle, neurotransmitters diffuse over the synaptic cleft and bind to receptors at the postsynaptic site triggering an action potential in the postsynaptic cell. Neurotransmitters are released in discrete amounts and cleared quickly from the synaptic cleft. To ensure that SV fusion is maintained during periods of high activity, SVs undergo a local cycle of tethering, docking, priming, fusion and endocytosis (see Figure 2).

In addition to SV mediated release of neurotransmitters, monoamines, growth factors and a wide variety of neuropeptides are secreted through the fusion of DCVs. Calcium dependent release of DCVs is not restricted to the synapse, but also occurs in dendrites, the soma and at extra-synaptic locations in the axon (De-Miguel and Trueta, 2005; Ludwig and Leng, 2006; Morris and Pow, 1991). Secreted neuropeptides are not cleared quickly from the extracellular space and can have half-lives up to 20 min in the brain (Ludwig and Leng, 2006; Mens et al., 1983). Therefore neuropeptides can exert their actions relatively far away from their release site. Neuropeptide release regulates many different processes, as diverse as memory formation, mood, appetite and social and reproductive behavior (Bartfai et al., 1988; Broberger and Hokfelt, 2001; Hokfelt et al., 2000; Hokfelt et al., 2008; Krieger, 1986; Meyer-Lindenberg et al., 2011; Ogren et al., 2010; Wiesenfeld-Hallin et al., 2002). The release of trophic factors like brain derived neurotrophic factor (BDNF, Dean et al., 2009; Hartmann et al., 2001; Kuczewski et al., 2008; Kuczewski et al., 2009; Kwinter et al., 2009; Lessmann and Brigadski, 2009), but also diffusible guidance cues (e.g. Semaphorin 3A; de Wit et al., 2006b; de Wit et al., 2009b) and transmembrane proteins (Breillat et al., 2007; Zylbersztejn et al., 2012) regulate neuronal

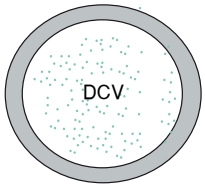
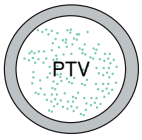
Vesicle type	Marker proteins	Cargo	Reference
 ~ 40 nm	SV2 syntaxin1 SNAP25 synaptotagmin1 synaptophysin Rab3	neurotransmitter	Zhang et. al., Neuron, 1998
 ~1 µm diameter	VAMP2 Rab3 PIP2 synaptotagmin1 synaptophysin VDCC amphiphysin	presynaptic components	Sytnyk et. al., J of Cell sci, 2004
 ~1 µm diameter	Rab11 transferrin	membrane components	Hsu et. al., Current op cell biol, 2010
 100 - 150 nm	Chromogranin Secretogranin	neuropeptides monoamines	Hsu et. al., Current op cell biol, 2010
 ~ 80nm	Bassoon Piccolo syntaxin SNAP25 N-Cadherin RIM munc13 munc18 VDCC	active zone proteins	Grace Zhai et. al., Neuron, 2001

Figure 1 Types of neuronal transport vesicles. Table with different types of neuronal transport vesicles: synaptic vesicles (SVs), synaptic vesicle precursors (SVPs), recycling endosomes (RE), dense core vesicles (DCVs) and Piccolo-Bassoon transport vesicles (PTVs). Approximate size and shape are displayed. The table shows the marker proteins by which these vesicles are classified and the cargo proteins they contain. VDCC: voltage-dependent calcium channel.

connectivity and trans-synaptic signaling, while proteolytic enzymes released from DCVs modulate the extra-cellular matrix and may play a role in synaptic plasticity (Frischknecht et al., 2008; Gualandris et al., 1996; Kwinter et al., 2009; Lochner et al., 2006; Silverman et al., 2005). Furthermore, mood and cognitive disorders, obesity and diabetes are all associated with neuropeptide dysfunction (Barchas and Elliott, 1986; Belzung et al., 2006; Fetissov et al., 2008; Hokfelt et al., 2003; Hokfelt et al., 2000; Holmes et al., 2003; Ogren et al., 2006; Valentino and Aston-Jones, 2010; Wiesenfeld-Hallin et al., 2002). Hence, it is clear that regulated DCV release is crucial in brain signaling.

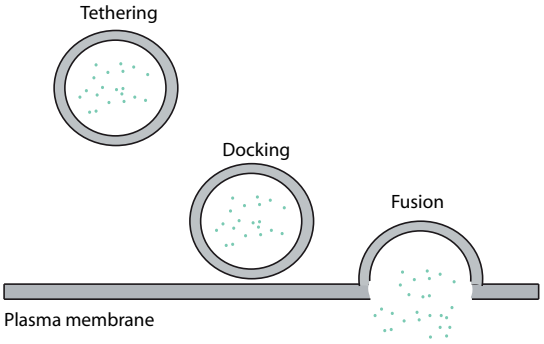


Figure 2 Scheme of different stages of vesicles at the plasma membrane. Tethered vesicles are in close proximity of, but not touching, the plasma membrane. During the docking stage, a vesicle becomes physically attached to the plasma membrane. Priming is the next step towards fusion, in which proteins like munc13 and CAPS undergo conformational changes to make the vesicles release-ready. Calcium influx activates calcium sensors like synaptotagmins and Doc2 that trigger vesicle fusion with the plasma membrane.

3 DENSE CORE VESICLES

DCVs were named after their dense core appearance in electron microscopic images. This dense core consists of condensed proteins that are efficiently packed in large amounts inside the vesicle. The ionic environment in DCVs causes the proteins to polymerize and aggregate into an insoluble condensed mass of proteins (Halban and Irminger, 1994).

3.1 Neuronal DCV cargo and their actions

DCVs secrete several important neuropeptides that are involved in many different processes in the brain. About 90 genes that encode precursors of peptides have been identified as neuropeptides according to the classical definition of a neuropeptide (see box 1). To illustrate their importance, some of the best-studied classes are described below.

3.1.1 Neurotrophic factors

Originally, neurotrophic factors have been implicated in the survival and differentiation of specific populations of neurons within the CNS (Lewin and Barde, 1996). More recently, release of neurotrophic factors in more mature systems has also been implicated in tuning synaptic transmission: for instance BDNF, functions as a retrograde signal secreted from dendrites that tunes presynaptic SV release (for review, see Lessmann et al., 2003). Hence, neurotrophic factors do not only function in trophic support of neurons, but also have other modulatory roles in the nervous system.

3.1.2 Guidance cues

DCVs also transport guidance cues, which are mainly involved in the development of the brain and are released from neurons to attract or repulse outgrowing axons to control the formation of proper synaptic contacts between brain regions (Kolodkin and Tessier-Lavigne, 2011). Also in the post-natal brain, release of guidance cues can shape connections. Semaphorins are among the best-studied axon guidance molecules (Pasterkamp and Kolodkin, 2003). In addition

Box 1

Neuropeptide gene family	Active peptide
Opiod gene family	Leu-enkephalin, Met-enkephalin, amidorphin, adrenorphin, peptide B, peptide E, peptide F, BAM22P, (α-MSH), γ-melanocytestimulating hormone (γ-MSH), β-MSH, ACTH, β-endorphin, α-endorphin, γ-endorphin, β-lipoprotein (β-LPH), γ-LPH, corticotropin-like intermediate peptide (CLIP), dynorphin A, dynorphin B, α-neo-endorphin, β-neo-endorphin, dynorphin-32, leu-morphin, nociceptin, orphanin FQ , neuropeptide1, neuropeptide 2
Vasopressin/ oxytocin gene family	Vasopressin (VP), neurophysin II, C-terminal glycopeptide CPP, Oxytocin (OT), neurophysin I (NP 1)
CCK/ gastrin gene family	Gastrin-34, gastrin-7, gastrin-4, CCK-8, CCK-33, CCK-58
Somatostatin gene family	SS-12, SS-14, SS-28, antrin, cortistatin-29, cortistatin-17
F- and Y-amide gene family	QRF-amide (neuropeptide RF-amide, GnIH, p518, RF-related peptide-2), RF-related peptide-1, RF-related peptide-3, neuropeptide VF, neuropeptide FF, neuropeptide AF, neuropeptide SF, NPY, C-flanking peptide CPON, PPY, PYY, PYY-(3-36), PrRP-31, PrRP-20
Calcitonin gene family	Calcitonin, katacalcin, Calcitonin gene related peptide I (α-CGRP), β-CGRP, IAPP (amylin, amyloid polypeptide), adrenomedullin, AM, PAMP, Adrenomedullin-2, intermedin-long (IMDL), intermedinshort (IMDS)
Natriuretic factor gene family	Atrial natriuretic factor (natriuretic peptide A, ANF, ANP, natriodilatine, cardiodilatine-related peptide), Brain natriuretic factor (natriuretic peptide B, BNF, BNP), C-type natriuretic peptide (CNP-23), CNP-29, CNP-53
Bombesin gene family	GRP-27, GRP-14, GRP-10 (neuromedin C), Neuromedin B (Ranatensin-like peptide, RLP)
Endothelin gene family	Endothelin-1, -2, -3
Glucagon/ secretin gene family	Glicentin; Glicentin related polypeptide (GRPP), oxyntomodulin (OXY) (OXM), glucagon, glucagon-like peptide 1 (GLP-1), GLP-2, secretin, VIP, PHM-27/PHI-27, PHV-42, PACAP-38, PACAP-27, PRP-48, GHRH (somatoliberin, GRF, somatocrinin, somatorelin, sermorelin), GIP (gastric inhibitory peptide, glucosedependent insulinotropic polypeptide)
CRH-related gene family	CRH, UNC-1, UNCII (stresscopin related peptide), UNC3 (stresscopin), urotensin-2
Kinin and tensin gene family	Substance P, neurokinin A (NKA, substance K, neuromedin L), neuropeptide K, neuropeptide gamma, neurokinin A, neurokinin B, neuromedin K
Neuromedins	Neuromedin S, neuromedin U
Tensins/ kinins	Bradykinin, kallidin, LMW-K-kinin, HMW-k-kinin, Angiotensin I, angiotensin II, angiotensin-(1–7), Neurotensin (NT), neuromedin N
Motilin family	Motilin, motilin associated peptide, Ghrelin, obestatin
Galanin family	Galanin, galanin message associated peptide (GMAP), Galanin-like peptide (GALP)
GnRH family	GnRH (LHRH, gonadoliberin), GnRH2
Neuropeptide B/ W family	Neuropeptide B-23 (peptide L7), neuropeptide B-29, neuropeptide W-23 (peptide L8), neuropeptide W-30, neuropeptide S
Insulins/ relaxins	Insulin, IGF-1/2, Relaxin-1, -2, -3
No family	TRH (thyroliberin), PTHrP-(1-36), PTHrP-(38-94), PTHrP-(107-139) (osteostatin), MCH, neuropeptide Glu-Ile (NEI), neuropeptide Gly-Glu (NGE), hypocretin-1 (orexin A), hypocretin-2 (orexin B), CART(1-39) CART-(42-89), AGRP, prolactin, Apelin-13, apelin-17, apelin-36 (APJ ligand, AGTRL1 ligand), Metastin (kisspeptin-54), (golgi transport 1 homolog A,golt1a), kisspeptin-14, kisspeptin-13, kisspeptin-10, diazepam-binding inhibitory protein
Cerebellins	Cerebellin-1, -2,-3, -4
Adipose neuropeptides	Leptin , Adiponectin, Visfatin-1, Resistin, Resistin-delta2, Resistin-like molecule-α/β/Y, Nesfatin-1, Beacon
Neurexophilins	Neurexophilins-1, -2, -3 -4
Granins	Chromogranin A/B, Secretogranin II/III, Secretory granule neuroendocrine protein-1, VGF, TLPQ-62, TLPQ-21, AQEE-30, LQEQ-19

Neuropeptide classes. Neuropeptide classes are shown as described on www.neuropeptides.nl. Neuropeptides are presented as the processed, biologically active products of the neuropeptide precursors and the encoding gene. The listed neuropeptides fall under the conservative definition of being expressed and released by neurons, and mediate or modulate neuronal communication by acting on cell surface receptors. Brain expression has been reported for the listed neuropeptides. The family of 'adipose neuropeptides' is an exception because they are released by adipose tissue and signal to the brain. The neuropeptide gene families are based on structural similarities or functional similarities.

to their well-established role in guiding axonal outgrowth during brain development, they continue to control inter-neuronal connections in the adult brain and play a role in refining synaptic efficacy (Fenstermaker et al., 2004; Gu et al., 2003; Tran et al., 2009; Yamashita et al., 2007). Hence, guidance cues have an important role in controlling synaptic plasticity in the adult nervous system besides their role in brain development.

3.1.3 Peptide hormones

Another class of neuropeptides encloses the peptide hormones which are released from the pituitary into the blood to act on organs in the body. The release of peptide hormones like oxytocin can influence animal behavior, like for example maternal behavior (Ludwig and Leng, 2006). Most peptidergic neurons also co-release a specific class of neurotransmitter and it is suggested that the release of neuropeptides after prolonged stimulation can influence the action of a neurotransmitter (Ludwig and Leng, 2006).

3.1.4 Opiod peptides

Opiod peptides are short sequences of amino acids which are processed from three opiod precursor genes; enkephalins, dynorphins (which includes β-endorphin) and proopiomelanocortin. Opiod peptides are involved in mood, motivation and pain and opiates are therefore used for pain medication (see for review, Kieffer and Gaveriaux-Ruff, 2002). Opiod peptides can also modulate neuronal activity on a smaller scale, as seen for dynorphin in the hippocampus. Dynorphin released from hippocampal granule cells reduces excitatory transmitter release from presynaptic terminals of the perforant path (Drake et al., 1994).

3.1.5 Monoamines

DCVs also contain monoamines (Liem and Copray, 1996; Llona et al., 1994). Monoamines are not peptides, but derive from aromatic amino acids like phenylalanine, tyrosine, and tryptophan, by the action of aromatic amino acid decarboxylase enzymes. The brain specific vesicular monoamine transporter 2 (VMAT2) localizes to DCVs that are released from the somato-dendritic compartment (Li et al., 2005). Although SVs also release monoamines, DCVs can contain combinations of monoamines, pack ten-times more monoamines than SVs and can be released from the somato-dendritic compartment (Hokfelt et al., 1980; Trueta et al., 2003). These properties suggest an important role for DCV release in retrograde signaling. In fact, the influence of dopamine on glutamate transmission is well established. Dopamine dampens the excitability of cortico-striatal synapses and thereby prevents overexcitation of the striatum (Bamford et al., 2004; Cepeda et al., 2001). Also serotonin can modulate glutamate transmission by controlling presynaptic output (Singer et al., 1996). In addition to the more local effects of monoamines, they can also influence the activity of other groups of cells via long-range paracrine effects and thereby regulate the receptiveness of the entire neuronal network (De-Miguel and Trueta, 2005).

These examples show that DCV cargo is involved in the regulation of many different brain processes. Different neuropeptides can be co-released from the same neuron working cooperatively or counteracting each other, creating an even more complex range of neuropeptide actions (see for review, van den Pol, 2012). Hence, DCV signaling is a central factor in brain communication on many different levels.

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3.2 Characteristics of neuronal DCV trafficking prior to release

SVs are enriched in synapses and locally recycle in response to activity. In contrast, DCVs are synthesized in the Golgi and transported from the soma to their release site. DCVs do not accumulate in terminals and therefore have to be recruited to these sites during stimulation, but how neurons maintain a steady supply of DCVs is still largely unknown. In vivo imaging at the Drosophila NMJ (neuromuscular junction) shows that DCVs are highly dynamic and that many DCVs pass release sites multiple times before some are captured and fuse with the plasma membrane. In these neurons, activity boosts the capture of DCVs and increases neuropeptide release (Shakiryanova et al., 2006). In mammals, similar activity-dependent mechanisms to capture DCVs at release sites may exist as in peptidergic neurons of the trigeminal ganglion. Moving DCVs stall after application of the phorbol ester PMA, which induces priming and increases DCV release (Sobota et al., 2010) and in cortical neurons, DCV transport is temporarily arrested during depolarization and calcium influx (de Wit et al., 2006b). Although we lack detailed understanding of the process that underlies activity dependent stalling of vesicle transport, most likely motor proteins or vesicle adaptor proteins are modified during stimulation. This may result in the complete detachment of DCVs from the microtubule track, which enables the interaction of DCVs with fusion-competent membrane patches where DCV fusion may occur.

3.3 Model systems to study DCV release

Because DCV cargo plays such an important role in brain communication it is crucial to understand how DCV release is regulated. In contrast to the detailed description of the molecular mechanisms that control SV release, our knowledge on the molecules that make up the release machinery of DCVs in neurons is very sparse. The release characteristics of DCVs and SVs are quite different. A relatively small pool of SVs is ready for immediate release upon action potential driven calcium influx. These vesicles are physically docked at the active zone and primed by the concerted action of priming proteins like Munc13, CAPS and RIM (Deng et al., 2011; Jockusch et al., 2007; Richmond et al., 1999). In contrast, DCVs are typically not pre-docked and hence not primed like SVs. Also, DCV release is not confined to the active zone and release requires more prolonged stimulation. Thus, these different release characteristics may indicate that DCV release uses different molecular machinery for release. So far, studies on DCV release have mainly focused on neuro-endocrine model systems and invertebrate neurons. The cargo released from different cell types that contain DCVs are listed in Figure 3.

3.3.1 Mammalian cell types

The best-studied mammalian model system for DCV release is the adrenal chromaffin cell. The adrenal gland consists of a cortex and a medulla and is specialized in bulk release of DCVs (de Diego et al., 2008). The cortex produces and secretes several corticosteroid hormones and secretion is controlled by hormones released from the pituitary. The chromaffin cells in the medulla are directly innervated by sympathetic neurons. Chromaffin cells originate from the neural crest and are therefore neuron-like cells with similar vesicle release machinery as found in central neurons. Studying vesicle release in chromaffin cells has resulted in an accurate understanding of the docking and priming mechanisms of these vesicles (de Wit, 2010; Liu et al., 2010).

The pancreas is another model system to study DCV release. The pancreas is of endodermal origin and consists of α- and Υ- cells that produce glucagon and pancreatic polypeptides and

cell type	cargo
chromaffin cells	catecholamines
beta cells	insulin
hypothalamic neurons	peptide hormones monoamines neuropeptides
pituitary neurons	peptide hormones
central neurons	monoamines neuropeptides growth factors

Figure 3 Cell types containing DCVs. DCVs are present in different cell types throughout the body and contain different cargo. Chromaffin cells are neuro-endocrine cells from the adrenal gland. Beta cells are of endodermal origin and are located in the pancreas. Neurons show some specialization, where pituitary and hypothalamic neurons are equipped to release hormones into the bloodstream, while central neurons release other modulatory factors into the brain itself.

β- and δ- cells that secrete respectively insulin and somatostatin. Pancreatic β-cells are most used to study DCV release. Insulin is crystallized inside the DCV and needs full opening of the fusion pore to diffuse out and dissociate into monomers. This makes β-cells very suitable to study the mechanism of fusion pore opening of DCVs (Rorsman and Renstrom, 2003).

3.3.2 Neuronal model systems

Neuronal DCV release has been studied in invertebrate neurons of the fruit fly Drosophila and the nematode C. elegans. These model systems have provided valuable knowledge on basic properties of DCV trafficking and release. Because of its high accessibility, the neuromuscular junction (NMJ) of Drosophila and C. elegans is a widely used model system to study neuronal DCV transport and release mechanisms. Elegant photobleaching studies on DCV dynamics at the NMJ revealed a strong correlation between DCV mobility and release probability. Electrical stimulation of NMJs leads to increased calcium release from internal stores that activates Ca^{2+}/calmodulin-dependent kinase II (CamKII), increases the mobility of presynaptic DCVs and induces post-tetanic potentiation of DCV release (Shakiryanova et al., 2007; Shakiryanova et al., 2011; Shakiryanova et al., 2005; Shakiryanova et al., 2006). In a recent study, the Levitan lab revealed that synapses are supplied with DCVs by sporadic capture of circulating DCVs. This mechanism of long-range transport in anterograde and retrograde direction with inefficient bi-directional capture is ideally suited to uniformly supply the sequential release sites in Drosophila NMJs (Shakiryanova et al., 2005; Shakiryanova et al., 2006; Wong et al., 2012). Whether such mechanisms act in mammalian central neurons and which protein machinery is involved is currently unknown.

A series of studies in C. elegans tested the role of the SV priming factors UNC-13 and UNC-31 (CAPS) in DCV docking and release. The Jorgensen lab showed that CAPS, but not UNC-13, is involved in DCV docking via interactions with the SNARE protein syntaxin (Hammarlund et al., 2008). Interestingly, deletion of CAPS, but not UNC-13, results in a strong reduction of DCV release (Speese et al, 2007). This suggests that in C. elegans, CAPS and UNC-13 serve parallel and unique roles in DCV and SV docking and release. While UNC-13 controls SV docking and release, CAPS controls the same steps for DCV exocytosis. In this thesis, the function of the mammalian orthologs of UNC-13 and CAPS in DCV release from hippocampal neurons will be addressed which reveals that in mammalian neurons both protein families play important roles in DCV release (Chapters 2 and 3).

In the mammalian brain, DCV release has been most thoroughly investigated in the neurohypophysis, where axons from magnocellular neurons from the hypothalamus release oxytocin and/or vasopressin into the blood (Sofroniew, 1980). Magnocellular neurons also release oxytocin and vasopressin from their dendrites and hence provide a suitable model system to study dendritic peptide release (Ludwig and Leng, 2006). Several key observations, for instance the importance of different activity patterns and calcium kinetics that regulate neuropeptide release, have been made using these neuronal preparations (Tobin et al., 2004). However, because magnocellular synapses are very large and contain many DCVs it is unlikely that similar trafficking and release mechanisms are being used by central neurons from the cortex and hippocampus because they are much smaller and generally not have many pre-docked DCVs. In this thesis, we use hippocampal cultures to investigate DCV release principles and investigate whether SV and DCV release are differentially regulated in the same neuron.

4 COMPARISON OF SV AND DCV RELEASE IN NEURONS

SVs and DCVs co-exist in neurons and are both released in response to calcium influx upon activity. Although the general principle of activity dependent release applies to both types of vesicles, the localization, type of stimulation, source of calcium, and membrane recruitment are quite different and will be discussed here.

4.1 Localization

Presynaptic terminals contain 100-300 SVs (Micheva and Smith, 2005; Schikorski and Stevens, 1997). A small subset of these vesicles, the readily releasable pool, is docked at the active zone. Upon arrival of an action potential, the resulting calcium influx triggers the fusion of vesicles from this pool (Rosenmund and Stevens, 1996). In contrast, DCVs are generally not pre-docked at the active zone in central neurons. In fact, at steady state, many synapses do not contain DCVs while the ones that do only harbor a few DCVs at any given time. Despite this, DCVs do fuse at synapses, but they also fuse extra-synaptically and in the somato-dendritic compartment (Xia et al., 2009). SVs are divided into several different pools according to their release state: the recycling pool, the total releasable pool and the readily releasable pool (Rizzoli and Betz, 2005). Whether DCVs can be subdivided into several pools with different release probabilities is currently unclear.

4.2 Calcium

DCV release is triggered by calcium influx from voltage gated calcium channels, just like SV release. Synapses contain mostly N, P and Q-type calcium channels, and SV release is regulated

through P/Q-type channels (Ertel et al., 2000; Mori et al., 1991; Williams et al., 1992). Previous work on release of dynorphin and BDNF from hippocampal neurons and oxytocin from magnocellular neurons has shown that somatic DCV release is mainly triggered by a global increase in calcium levels via non-synaptic L-type calcium channels (Simmons et al., 1995; Tobin et al., 2011; Xia et al., 2009). Furthermore, release of calcium from intracellular calcium stores can also influence DCV release as is explained in more detail below.

4.3 Mobility

As mentioned above (section 3.3.2), calcium influx in Drosophila NMJs strongly increases synaptic DCV mobility and undocked DCVs appear to fuse first (Shakiryanova et al., 2005). DCV release is increased after neuronal activity, suggesting that mobilization of DCVs is important for fusion. Brief synaptic activation can increase DCV release for up to minutes (Shakiryanova et al., 2007). Calcium influx activates ryanodine receptors on intracellular calcium stores, which then release more calcium. This calcium-induced calcium release (CICR) creates calcium waves in the cytosol and is an important factor in sustaining DCV mobilization and release (Ludwig and Leng, 2006). SV mobility also increases upon calcium influx where synapsin regulates the release of SVs from actin bundles in the synapse stimulating SV release (Chi et al., 2001). Synaptic terminals can also share recycling SVs (Darcy et al., 2006). All these SV mobilization mechanisms are actin dependent, while DCV mobilization seems to be more dependent on tubulin.

4.4 Stimulation paradigms

It is well established that DCV secretion requires more intense stimulation than SV secretion (see Hartmann et al, 2001 and Matsuda et al, 2009). This intense stimulation might produce bulk Ca^{2+}-elevations and trigger CICR from internal calcium stores. In magnocellular neurons, calcium release from internal stores induces DCV mobilization, moving the DCVs closer to the dendritic plasma membrane, thereby increasing their release probability (Tobin et al., 2004). Importantly, several studies have addressed the importance of the length and repetitiveness of stimuli to trigger efficient DCV release from different locations in the neuron (Hartmann et al., 2001; Matsuda et al., 2009).

4.5 Recruitment

SVs undergo a local and relatively fast cycle of exocytosis followed by endocytosis to sustain release during prolonged stimulation. In contrast, DCVs do not accumulate in terminals and are likely recruited from a dynamic pool. DCV endocytosis probably does not play a significant role in the maintenance of DCV release because of the large supply of newly arriving DCVs (Bauer et al., 2004; Tsuboi et al., 2004). We have previously shown that DCVs are actively transported along microtubules and arrest upon calcium influx (de Wit et al., 2006b). This may trigger the detachment from these tracks, but how these vesicles are recruited to release sites is unknown.

Thus, although both vesicle types co-exist in neurons and show calcium dependent release, the action of their cargo, their location and mode of release are very different. Proteins involved in SV tethering, docking and release may also be important for DCV recruitment and release but the differences between SVs and DCVs described in this paragraph predict that different isoforms and/or additional proteins likely play a role.

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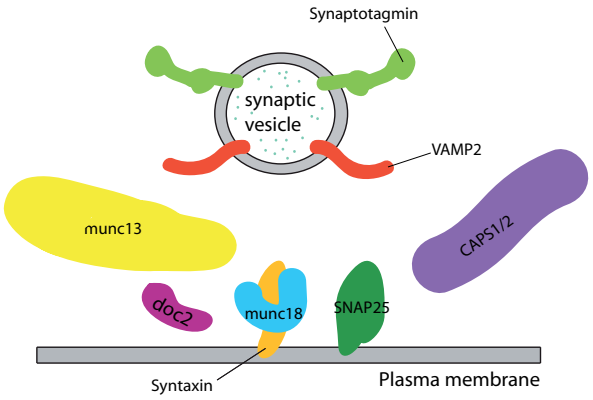
5 VESICLE RELEASE

Although SV and DCV release characteristics are very different, they both show activity dependent release at the same target membrane. The well-investigated SV release machinery may thus provide some interesting leads for the study of DCV release principles.

5.1 The synaptic vesicle cycle

The SV cycle consists of several steps. Vesicles are first tethered to the membrane before they physically dock at the active zone. The next priming step generates release ready vesicles. From this readily releasable pool, calcium influx triggers the fusion of vesicles (see Figure 4).



protein	spontaneous	evoked	lethality
Syntaxin-1	√	√ LTP x	
VAMP2	x	x	†
SNAP25	√	x	†
munc18-1	x	x	†
munc13-1/2	x	x	†
CAPS-1/2	x	x	†
Synaptotagmin-1	√	x	†
Doc2a/b	x	√	

x no release
√ release
† lethal

Figure 4 Proteins involved in SV release. Priming proteins CAPS-1/2 and munc13-1/2 are present in the synaptic terminal. SNARE proteins syntaxin-1 and SNAP25 are on the plasma membrane, while VAMP resides in the SV membrane. The calcium sensor synaptotagmin-1 is also present on the vesicle membrane, while the high affinity calcium sensor Doc2 can attach to lipids, but is cytoplasmic under resting conditions. The accessory protein munc18 is cytoplasmic but has a high affinity for its binding partner syntaxin-1 and forms docking-acceptor complexes on the plasma membrane. The figure shows a subset of the proteins involved in SV release. The table shows the effect of null mutations of the genes involved in the SV cycle on the spontaneous and evoked release of SVs. LTP = long term potentiation, Red x = abnormal SV release, green mark = normal SV release. Red cross marks the lethality of the knockout mice.

5.1.1 Docking of SVs

Docking of SVs to the plasma membrane is regulated by munc18, SNAP25 and synaptotagmin. The S/M protein munc18 associates with the SNARE protein syntaxin and with the assembled SNARE complex. Munc18 null mutant neurons are completely silent with no spontaneous or evoked release (Toonen and Verhage, 2007; Verhage et al., 2000) and levels of munc18 influence the number of docked SVs (Toonen et al., 2006). In addition, high pressure freezing of hippocampal synaptic terminals shows that munc13 deficient mice have a defect in SV docking (Siksou et al., 2009) and synaptotagmin IV (SytIV) influences docking probably through an interaction with the motor protein KIF1A (Arthur et al., 2010). How these proteins interact with each other in regulating SV docking is not clear. Because they are all interacting partners of syntaxin, this protein might be an important converging point for regulating SV docking. However, using classical chemical fixation methods in neurons, no effect on vesicle docking was observed upon deletion of syntaxin (de Wit et al., 2006a).

5.1.2 Priming of SVs

The three main priming proteins for SV release are RIM, munc13 and CAPS. Deletion of the two major Munc13 genes expressed in brain, results in a nearly complete absence of spontaneous and evoked release (Varoqueaux et al., 2002). Deletion of CAPS1 or both CAPS isoforms in mice is lethal and these mice show severe defects in spontaneous and evoked SV release (Jockusch et al., 2007). This shows that vesicle priming is an essential step in SV exocytosis. Munc13 and CAPS both regulate the release readiness of SVs, most likely by their interaction with syntaxin. Although their function in SV release seems similar, munc13 overexpression cannot rescue the CAPS null mutant phenotype and vice versa (Jockusch et al., 2007). Conditional knockout of both RIM genes severely impairs the Ca^{2+} responsiveness and synchronization of SV release (Kaeser et al., 2012). RIM proteins recruit and tether calcium channels to the active zone, and also anchor and regulate munc13 at synapses. The interaction of RIM with munc13 prevents the inhibitory auto-homodimerization of munc13, which enables monomeric munc13 to contribute to the priming of SVs (Deng et al., 2011).

5.1.3 Calcium regulators of SV release

Calcium sensing proteins play a crucial role in the fusion process. These proteins bind calcium and interact in a calcium dependent way with the membrane lipids and the SNARE complex to overcome the last barrier before fusion. Synaptotagmin-I (SytI) is the main calcium sensor in evoked SV release and knockout mice show a severe defect in synchronous release (Geppert et al., 1994). The synaptotagmin protein family consists of 17 synaptotagmin isoforms, with different affinities for calcium and interacting proteins, which provides synapses with different calcium sensitivities (Ullrich et al., 1994). Doc2, a cytosolic calcium sensor with much higher calcium affinity than SytI, is the major sensor for spontaneous release of SVs (Groffen et al., 2010).

5.1.4 Fusion of SVs

SNARE proteins regulate the fusion of two opposing membranes. The zippering of the four-helical SNARE bundle that consists of the coiled coil domains of syntaxin-1, SNAP25 and VAMP2 drives the fusion of SVs with the plasma membrane. Syntaxin-1 and SNAP25 are both present on the target membrane, while VAMP2 is anchored in the SV membrane (Hanson et al., 1997; Rizo and Sudhof, 2002). VAMP2 null mutant mice have a lethal phenotype and show a severely

reduced evoked response and a decrease in spontaneous release (Schoch et al., 2001). Deletion of SNAP25 is also lethal and these mice show impaired evoked release, but normal spontaneous release (Washbourne et al., 2002). Surprisingly, syntaxin-1a knockout mice are viable and only show abnormalities in long term potentiation, probably because the amount of syntaxin-1b is enough to support basic synaptic transmission (Fujiwara et al., 2006).

5.2 Steps leading to DCV release

In contrast to our detailed knowledge of the proteins involved in the different steps of SV release, many fundamental questions remain for DCV release. The mechanistic knowledge on DCV release so far is described here.

5.2.1 Docking of DCVs

In the neuromuscular junction of C. elegans, CAPS proteins are involved in docking DCVs. Overexpression of open syntaxin bypasses this requirement. Hence, CAPS likely functions to promote the opening of syntaxin-1, and thereby promotes vesicle docking (Hammarlund et al., 2008). Although munc13 and CAPS share an important MUN domain, *unc-13* deletion only has a minor effect on DCV docking (Hammarlund et al., 2008). So, in C. elegans neurons, syntaxin and CAPS appear to work together as a docking platform for DCVs. Deletion of munc13-1 in chromaffin cells does not affect the docking of DCVs (Ashery et al., 2000). The orthologs of munc18 are essential for synaptic transmission in C. elegans (*unc-18*) and Drosophila (*Rop*), but these mutants have not been used to study DCV release (Harrison et al., 1994; Miller et al., 1996; Schulze et al., 1994). In adrenal chromaffin cells, the minimal DCV docking machinery comprises of SytI on the vesicle membrane and the Munc18/Syx/SNAP25 acceptor complex on the plasma membrane (de Wit et al., 2009a). Whether these proteins have similar roles in docking DCVs in central neurons is not yet known.

5.2.2 Priming of DCVs

CAPS was identified as a cytosolic protein involved in DCV release in neuro-endocrine cells (Walent et al., 1992). Deletion of UNC-31 (CAPS) in Drosophila results in a strong defect in DCV release (Renden et al., 2001). In addition, CAPS1 affects catecholamine loading of DCVs in chromaffin cells (Speidel et al., 2005). In mouse hippocampal neurons, CAPS2 increases BDNF release when overexpressed on a CAPS2 knockout background (Shinoda et al., 2011). Munc-13, while absent from chromaffin cells, strongly stimulates release when overexpressed in these cells (Ashery et al., 2000). Therefore, Munc13 and CAPS seem to function as important priming factors for DCVs in these model systems.

5.2.3. Calcium regulators of DCV release

It is not yet known which calcium sensors function in neuronal DCV release. Doc2 proteins do not have orthologs in Drosophila or C. elegans (Fukuda, 2003), and null mutants of Syt-I have not been investigated for DCV release. Data from neuro-endocrine cells suggest that several Syt isoforms and the cytoplasmic calcium sensor Doc2 are good candidates for DCV release in neurons. In PC12 cells, SytI is localized to DCVs and functions together with SytIX in exocytosis of DCVs (Lynch and Martin, 2007). Furthermore, SytV was found to specifically localize to DCVs in PC12 cells and mouse brain. This suggests that SytV may be a DCV specific calcium sensor (Saegusa et al., 2002). In chromaffin cells, release is abolished in SytI and SytVII

double null mutants (Schonn et al., 2008; Sorensen et al., 2003a; Voets et al., 2001). Doc2 has not been studied in chromaffin cells, but overexpression of Doc2 enhances DCV release in PC12 cells (Orita et al., 1996). Furthermore, a Doc2 mutant that is unable to bind syntaxin-SNAP25 heterodimers decreases the number of docked DCVs in PC12 cells (Sato et al., 2010).

5.2.4 Fusion of DCVs

In chromaffin cells, the cleaving of individual components of the SNARE machinery with neurotoxins, results in a strong decrease in DCV release (Hohne-Zell et al., 1994; Xu et al., 1998). In addition, SNAP25 null mutant chromaffin cells have a severe defect in DCV release (Sorensen et al., 2003b) and double null mutants of VAMP1 and VAMP2 show completely abolished DCV release (Borisovska et al., 2005). Hence, these data from chromaffin cells strongly indicate a vital role for the canonical SNARE complex in mediating DCV release. However, data on the involvement of these SNARE proteins in DCV release from neuronal systems in mammals, C. elegans and Drosophila is lacking.

Hence, it is beyond doubt that DCV signaling is a central factor in brain communication. However, in contrast to our detailed knowledge of SV secretion mechanisms, many fundamental questions on DCV trafficking and secretion remain.

6. AIM OF THIS THESIS

DCV release plays an important role in the nervous system, but we understand surprisingly little of the machinery that governs DCV release from central neurons. Although SVs and DCVs are both present in the nervous system and both show activity dependent release, their cargo, size, localization and timing of fusion are very different. This suggests that in addition to the canonical SV release machinery proteins, other proteins may modulate DCV transport and fusion. The main aim of this thesis is therefore to provide mechanistic insight in neuronal DCV release. To this end, we use null mutants of the proteins involved in SV release and analyze the similarities and differences between SV and DCV release in mammalian neurons.

In **chapter 2** we investigate the involvement of the classical SV priming factor munc13-1 in DCV release. We find that DCVs are released both at synaptic sites as well as extra-synaptic sites. Detailed comparison of synaptic versus extra-synaptic DCV release, revealed that DCVs fuse preferentially at synaptic sites and that synaptic release requires less robust stimulation compared to extra-synaptic release. We tested the effect of the synaptically localized priming factors munc13-1 and munc13-2 on fast synaptic DCV release. We found that munc13-1/2 facilitate DCV fusion but, unlike for SV release, are not essential for DCV release. In addition, munc13 emerged as an important regulator of synaptic preference of DCV release and ectopic overexpression of munc13-1 is sufficient to make extra-synaptic release as efficient as synaptic release.

In **chapter 3** we investigate the role of the other major SV priming factor CAPS in DCV release. We studied the effect of CAPS-1 and CAPS-2 single and double deletion on DCV release. We first show that in contrast to catecholamine loading problems in CAPS1 null mutant chromaffin cells, CAPS deletion does not affect peptide loading in neurons. Vesicle release in CAPS-1/2 double null mutant neurons is strongly affected, but in contrast to munc13, CAPS does not alter

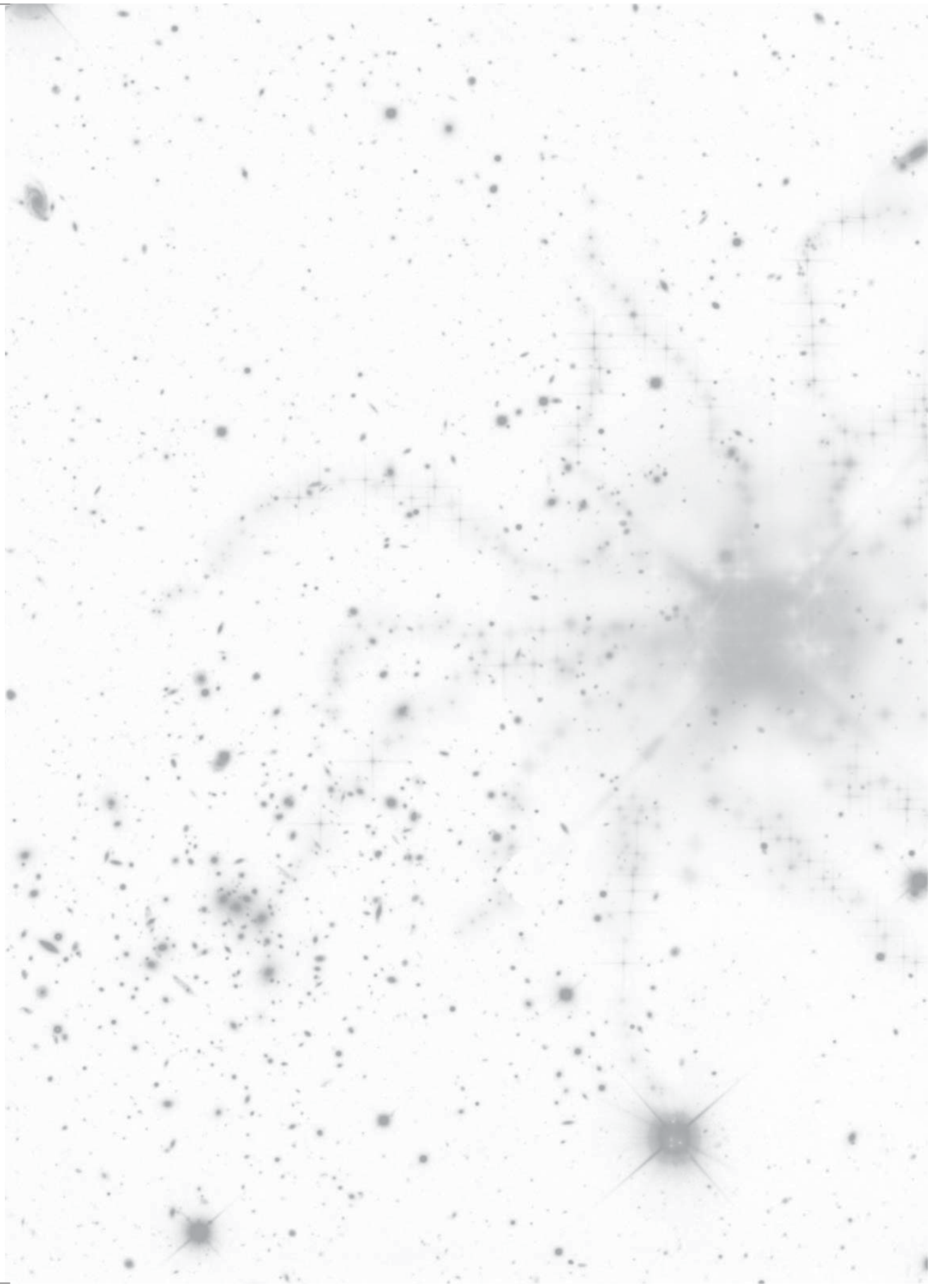
synaptic preference of DCV release. Hence, CAPS-1 and CAPS-2 regulate release readiness of DCVs independent of their location.

In **chapter 4** we investigate whether the SM protein munc18-1 is essential for neuronal DCV release. All types of vesicle fusion require the combined action of SNARE complexes and SM proteins. Therefore we tested the hypothesis that munc18-1 is involved in DCV release. Because munc18-1 null mutant neurons survive until 4 days in vitro, we also studied the differences between developing and mature neurons. We show that the total number of DCVs in developing neurons is reduced but that DCV release probability is similar compared to mature neurons. Interestingly, deletion of Munc18-1 did not abolish DCV release in developing neurons. This suggests that during development other Munc18 isoforms function in supporting calcium and SNARE dependent DCV release.

In **chapter 5** we test which SNARE proteins are involved in DCV release. Although SNARE proteins regulate most if not all fusion processes, we do not know which SNARE complex drives DCV release. We tested this using neurotoxins that cleave specific isoforms of the SNARE proteins. Furthermore, we used SNAP25 null mutant neurons, to identify the SNAP isoform involved in DCV fusion. We found that DCVs use at least two different SNARE complexes, depending on the developmental stage or location of release.

Chapter 6 studies the calcium sensors involved in DCV release. We compare two calcium sensors with different calcium affinities: Synaptotagmin-1, the vesicular low affinity calcium sensor that drives synchronous SV release, and Doc2, the cytosolic high affinity calcium sensor for spontaneous SV release. We found that DCV release is not affected by deletion of Syntapotagmin-1, but analysis of Doc2a/b double null mutant neurons shows that Doc2 plays an important role in DCV release. The cytosolic location of Doc2 in combination with its high affinity for calcium might provide a calcium-sensing mechanism for DCVs that are distant from the plasma membrane.

Chapter 7 summarizes the main findings, discusses the results and provides a detailed working model of the DCV release machinery in mammalian hippocampal neurons.



CHAPTER 2

MUNC13 CONTROLS THE LOCATION AND EFFICIENCY OF DENSE-CORE VESICLE RELEASE IN NEURONS

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ABSTRACT

Neuronal dense-core vesicles (DCVs) contain diverse cargo crucial for brain development and function, but the mechanisms that control their release are largely unknown. We quantified activity-dependent DCV release in hippocampal neurons at single vesicle resolution. DCVs fused preferentially at synaptic terminals. DCVs also fused at extra-synaptic sites but only after prolonged stimulation. In *munc13-1/2* null mutant neurons, synaptic DCV release was reduced but not abolished and synaptic preference was lost. Remaining fusion required prolonged stimulation, similar to extra-synaptic fusion in wildtype neurons. Conversely, Munc13-1 overexpression promoted extra-synaptic DCV release, also without prolonged stimulation. Thus, Munc13-1/2 facilitate DCV fusion but, unlike for synaptic vesicles, are not essential for DCV release and Munc13-1 overexpression is sufficient to produce efficient DCV release extra-synaptically.

INTRODUCTION

Neuronal dense-core vesicles (DCVs) contain neuropeptides, neurotrophic factors, monoamines and other modulatory substances that are essential during brain development and regulate synaptic plasticity in the adult brain (Huang and Reichardt, 2001; McAllister et al., 1999; Poo, 2001; Samson and Medcalf, 2006). To date, more than 50 biologically active peptides have been identified that have profound effects on brain and body function. Not surprisingly, dysregulation of DCV signaling is associated with many diseases such as mood and cognitive disorders, obesity and diabetes (Meyer-Lindenberg et al., 2011). DCVs are filled with cargo at the Golgi network (Kim et al., 2006) and transported through axons and dendrites via microtubule-based motors of the kinesin and dynein families (Hirokawa et al., 2009; Schlager and Hoogenraad, 2009). Calcium influx triggers DCV release from synaptic and extra-synaptic regions on axons and dendrites (de Wit et al., 2009b; Hartmann et al., 2001; Knobloch et al., 2012; Ludwig and Leng, 2006; Matsuda et al., 2009) and previous work identified several stimulation paradigms that trigger DCV fusion (Bartfai et al., 1988; Hartmann et al., 2001; Matsuda et al., 2009; Verhage et al., 1991). Yet, little is known about the molecules that control the location and kinetics of DCV secretion in mammalian neurons.

In contrast to the relative lack of understanding of DCV fusion, synaptic vesicle (SV) fusion mechanisms are much better understood (Jahn and Scheller, 2006; Sudhof and Rothman, 2009). SVs cluster at active zones, fuse upon calcium influx and locally recycle at the presynaptic terminal (Sudhof, 2004). DCVs are generally more dynamic (de Wit et al., 2006; Matsuda et al., 2009; Shakiryanova et al., 2006) and are present in synaptic terminals as well as neurites and perikarya but it is not clear whether DCVs specifically cluster at synaptic terminals or use activity-dependent recruitment mechanisms as seen in *Drosophila* neuromuscular junctions (Shakiryanova et al., 2006; Wong et al., 2012). Hence, transport and release characteristics of SVs and DCVs differ to some extent, but they also share properties such as the requirement for functional SNARE complexes and calcium influx to initiate fusion (Xu and Xu, 2008). Therefore, proteins that function in synaptic vesicle release (de Wit et al., 2009a; Garner et al., 2000; Rosenmund et al., 2003; Sorensen, 2009; Verhage and Toonen, 2007) may have similar functions in DCV release in neurons. Yet, no candidates have been investigated to date.

To assess the spatial and temporal characteristics of neuronal DCV release in a quantitative way, we used an optical probe to visualize single DCV release events in hippocampal neurons. We found that DCVs are preferentially released from synaptic terminals. Release onset is faster and the rate of release is higher at synapses compared to extra-synaptic sites. To unravel underlying molecular mechanisms we investigated the role of the Munc13 family members Munc13-1 and -2, SV priming proteins that are essential for SV release (Augustin et al., 1999; Basu et al., 2007; Rhee et al., 2002; Varoqueaux et al., 2002). We show that deletion of Munc13-1/2 strongly decreased but did not abolish DCV release, while Munc13-1 over-expression resulted in increased DCV release. Strikingly, both the absence and over-expression of Munc13 changed the synaptic preference of DCV release: Munc13 deletion specifically reduced synaptic release events, while over-expression increased release only from extra-synaptic sites. Thus in addition to its fusion promoting role, Munc13 also affects the localization of DCV release and Munc13 overexpression is sufficient to promote efficient DCV release at extra-synaptic sites along the plasma membrane.

RESULTS AND DISCUSSION

Optical sensor for DCV release in neurons

To study neuronal DCV release, we used reporters generated by fusing the canonical DCV cargo proteins Neuropeptide Y (NPY) or Semaphorin-3a to pH-sensitive EGFP (pHluorin) (Fig. 1A and Fig. S1). We have previously shown that these cargo, when overexpressed in mouse hippocampal neurons, are co-expressed in the majority of DCVs with an almost 90% overlap with endogenous DCV cargo Chromogranin A (Fig 1B and, de Wit et al., 2009b). DCV-pHluorin release events become visible as bright diffraction limited spots (Fig. 1C). To test whether these spots represent single or multiple DCV release events, the fluorescence intensity increase (ΔF) of individual release events was compared to the ΔF of DCV puncta upon NH_4^+ superfusion (to de-quench intra-vesicular pHluorin, Fig. 1D). The ΔF intensity plot upon NH_4^+ application showed a skewed distribution with a major population at ± 6 a.u. that overlapped with the ΔF of individual release events, suggesting that the latter are generally single vesicle fusion events (Fig. 1D).

To initiate release of DCVs, neurons were stimulated with 16 bursts of 50 action potentials (AP) at 50 Hz. This stimulus is optimal for release of the neuropeptide BDNF from hippocampal neurons (Hartmann et al., 2001). DCV release was strongly coupled to this stimulation and resulting calcium influx (Fig. 1E and Fig. S1). Only a few DCVs were released before or after the stimulus train (Fig. 1E-F and Fig. S1). Other stimulation paradigms were not as effective in releasing DCVs (Fig. S1).

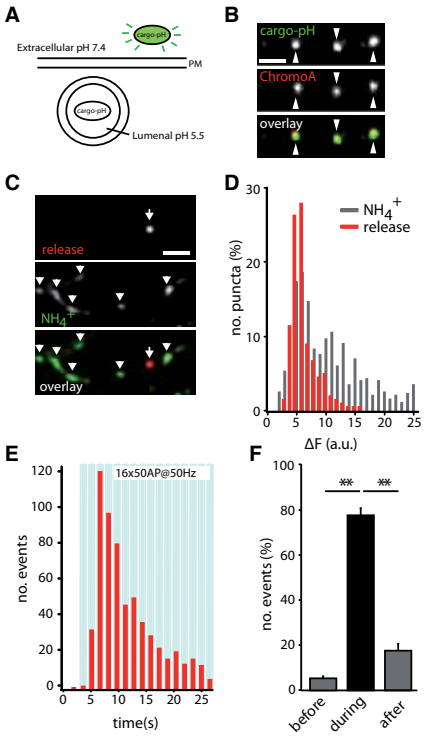

Figure 1 Optical sensor for DCV release in neurons. (A) Schematic representation of an optical reporter for dense-core vesicle release that allows visualization of single DCV fusion events. (B) Confocal image of a neuron transfected with sema3A-pHluorin (cargo-pH) and stained for endogenous DCV cargo chromogranin A showing complete overlap (arrowheads). Scale bar, 2 µm. (C) Image series showing a cargo-pH release event (arrow) and the NH_4+ response to reveal all vesicles in the neurite (arrowheads). (D) Normalized frequency distribution of ΔF for DCV release events measured with Sema3a-pHluorin (red bars) and ΔF upon NH_4+ perfusion (grey bars) (NH_4+: 395 puncta, 7 cells; release: 293 puncta, 30 cells, median ΔF release = 5.4 a.u.; median ΔF NH_4+ = 6.0 a.u.). (E) Frequency distribution of DCV release events measured with Sema3a-phluorin (570 release events in 53 cells; blue bars, 16 bursts of 50 AP at 50 Hz). (F) DCV release events during 60 seconds before stimulation, during stimulation and during 70 seconds after stimulation (before: 1 ± 0.6; during: 16 ± 3.4; after: 5 ± 1.3 vesicles/cell, n = 21 cells, N = 3).

DCVs do not accumulate at synapses but DCV release is enriched and more efficient at these sites

Many proteins involved in SV fusion are enriched in synaptic terminals (Sudhof, 2004). If these proteins were also involved in DCV release, DCV release should be enriched at synaptic sites. To test this, we quantified synaptic and extra-synaptic release (Fig. 2A-B) using the SV protein synapsin to visualize synapses in living neurons (Gitler et al., 2004). Synapsin-mCherry fusion proteins formed discrete puncta along the dendrite that co-localized with endogenous VAMP2, confirming that synapsin-mCherry is correctly localized to synapses (Fig. S1F and our previous results, de Wit et al., 2006).

We first tested whether DCVs accumulate at synapses. In fixed neurons, 18.7 ± 3.1 % of synapsin-labeled terminals overlapped with DCV-pHluorin puncta (n = 616 synapses measured in four cells). Similar results were obtained for the endogenous cargo Secretogranin II (19.9 ± 3.7 %, n = 493 synapses in 5 cells). Hence, in hippocampal neurons most synapses do not contain DCVs.

As DCVs are smaller than our resolution limit, individual DCV puncta may contain multiple DCVs. To assess this, we compared fluorescence intensity of individual puncta upon NH_4^+ application at synapses and extra-synaptic regions. DCV-pHluorin puncta showed a similar intensity distribution at synapses as outside synapses with a major population at 6 a.u. (Fig. 2C-D, synaptic: median 6.0, average 7.4 ± 0.8, n = 589 puncta; extra-synaptic: median 5.7, average 7.0 ± 0.8, n = 612 puncta, N = 8 cells, $p > 0.05$). Thus, the majority of single puncta are indeed single DCVs (compare Fig. 1D with Fig. 2C-D), while some puncta comprise multiple DCVs (between 1-4 with 6 a.u. representing a single DCV, see Fig. 1D). These DCV clusters are present at extra-synaptic as well as synaptic sites with no specific enrichment in synapses. Electron micrographs corroborated these findings and revealed both single DCVs and DCV clusters outside synaptic areas (Fig. S2D). On average 1.4 DCV was present per synapse section (average DCV diameter: 68.9 ± 1.0 nm, Fig. 2E-F) and almost half of the sections did not contain DCVs (Fig. 2G). In sections with DCVs, most DCVs were randomly distributed in the terminal, while ± 22 % were physically attached (docked) to the active zone (Fig. 2G, inset). Together, these data show that most synapsin-labeled terminals do not contain DCVs and that DCV clusters are not enriched in terminals at rest.

Yet, the majority of DCV release events occurred at synapsin positive regions (synaptic: 66%, 315 events; extra-synaptic: 34%, 165 events, n = 34 cells, N = 3, Fig. 2H) and DCV release rates were much slower at extra-synaptic sites than at synapses (synaptic: 12.6 ± 0.3 vesicles/sec; extra-synaptic: 7.8 ± 0.2 vesicles/sec, $p < 0.05$, Fig. 2I). In line with this observation, synaptic DCV release required less intense stimulation compared to extra-synaptic release: Already after the first 50 APs, DCVs were released at synapses whereas extra-synaptic release started only after 3 bursts of 50 APs (Fig. 2I-J). Apart from the kinetic difference, no differences were observed in the size and shape of the individual release events (Fig. 2K). Hence, DCV release probability at synapses is higher than outside these regions.

Deletion of Munc13-1 reduces and delays synaptic DCV release

To gain insight into the molecular priming mechanisms of neuronal DCV release, we tested the role of the SV priming proteins of the Munc13 family in munc13-1/2 null mutant (Munc13 DKO) mice. In Munc13 DKO neurons, the number of DCVs per cell was not changed (Fig. S2A) but DCV release was reduced by more than 60% compared to WT (WT: 11.8 ± 2.3, n = 41 cells; Munc13 DKO: 4.7 ± 1.0, n = 23 cells, ** $p < 0.01$, Fig. 3A). In addition, DCV release rates were slower

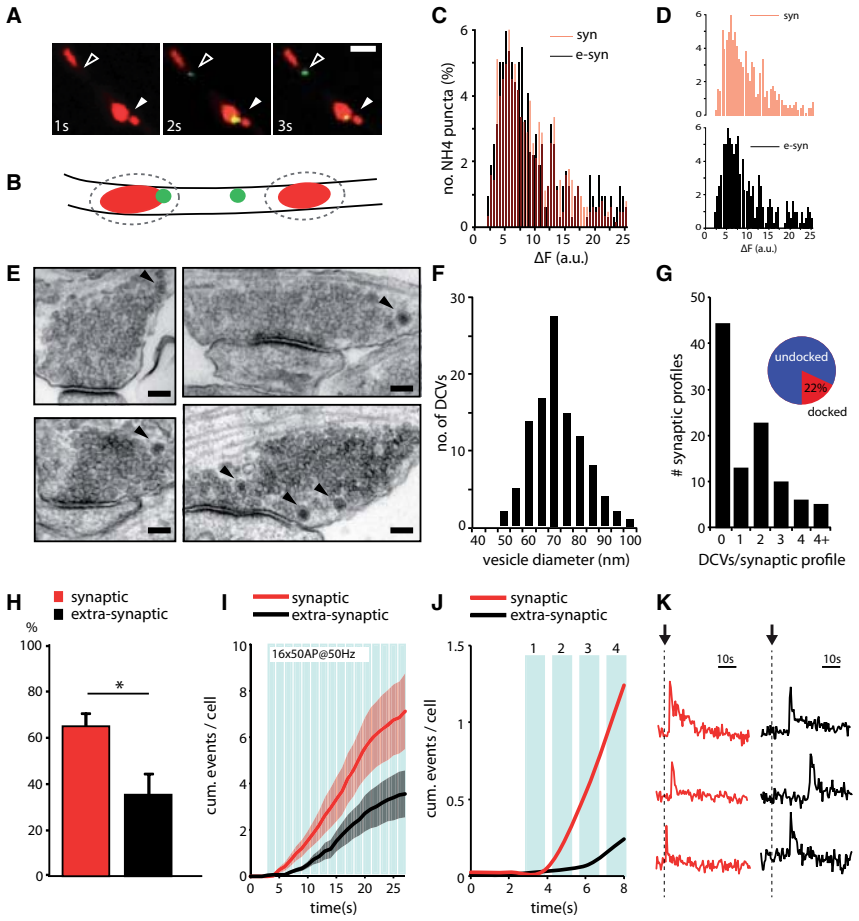

Figure 2 DCVs release is enriched and more efficient at synapses although DCVs do not accumulate at synaptic regions. (A) Example of a synaptic (filled arrowhead) and an extra-synaptic release event (open arrowhead), synapsin-mCherry shown in red and release events in green (scale bar, 1 µm). (B) Cartoon showing a synaptic (left green dot) and extra-synaptic (right green dot) release event. (C) Overlay of frequency distribution of ΔF upon NH_4+ application for synaptic DCV puncta (red bars, synaptic 589 puncta, syn) and extra-synaptic DCV puncta (black bars, extra-synaptic 612 puncta, e-syn). (D) Individual frequency distributions of ΔF upon NH_4+ application. (E) Electron-micrographs of synaptic terminals that harbor DCVs. Arrowheads point to DCVs, scale bar, 100 nm. (F) Diameter of DCVs in electron micrographs (average diameter: 68.9 ± 1.0 nm, 146 DCVs in 7 cells). (G) Number of DCVs per synaptic profile (102 synapses in 7 cells). Inset, ± 22 % of synaptic DCVs is docked to the plasma membrane (undocked 113, docked 33, total 146). (H) Percentage of synaptic and extra-synaptic DCV release events measured with Sema3a-pHluorin (235 synaptic release events, 131 extra-synaptic release events, n = 34 cells, N = 3, * p < 0.05). (I) Cumulative number of release events per cell for synaptic and extra-synaptic events (Synaptic release rate: 0.26 ± 0.03 vesicles/s; extra-synaptic: 0.13 ±0.02 vesicles/s). (J) Cumulative number of events during the first four bursts (blue bars 1, 2, 3 and 4 represent 50 AP at 50 Hz) for synaptic and extra-synaptic events. (K) Example traces of synaptic (red) and extra-synaptic (black) release events. Arrows mark start of the stimulation.

(WT: 0.73 ± 0.12 vesicles/s; Munc13 DKO: 0.32 ± 0.1 vesicles/s, * p < 0.05, Fig. 3B) and release required more prolonged stimulation as Munc13 DKO neurons released DCVs only after the third burst of 50 APs (Fig. 3B-C). Hence, deletion of Munc13-1/2 strongly reduced the number and rate of DCV release events. In contrast to SV release however, it did not totally abolish DCV release. *Munc13-1* single null mutant neurons showed a similar strong reduction of DCV release, indicating that Munc13-1 is the dominant isoform supporting DCV release (Fig. S3).

Munc13-1 is highly enriched in presynaptic terminals (Kalla et al., 2006) and could thus influence the localization of DCV release. We first excluded that deletion of Munc13-1 affected DCV localization in the synapse using electron microscopy (Fig. S3C, D). Next, using synapsin-mCherry as a synaptic marker we found that Munc13-1/2 deletion did not change the localization of DCVs prior to release (Fig. S2C and Fig. 3D) but strongly affected the ratio of synaptic versus extra-synaptic release (Fig. 3E) by reducing the number of synaptic release events (Fig. 3F). This suggests that Munc13-1 specifically promotes synaptic DCV release. Consistent with this observation, Munc13-1/2 deletion affected synaptic DCV release rates much more than extra-synaptic release rates (WT synaptic: 0.49 ± 0.09 vesicles/s; Munc13 DKO synaptic: 0.17 ± 0.1 vesicles/s, p < 0.05; WT extra-synaptic: 0.24 ± 0.08 vesicles/s; Munc13 DKO extra-synaptic 0.16 ± 0.13 vesicles/s, Fig. 3G-H).

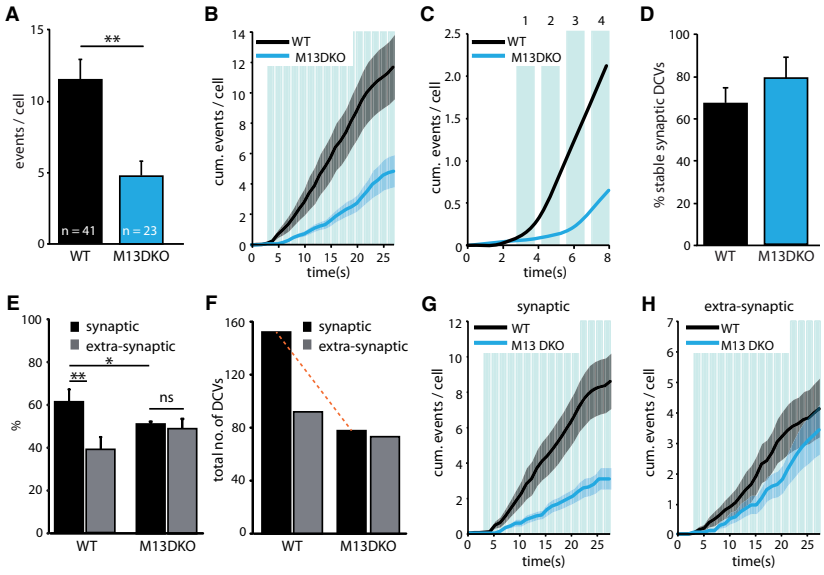

Figure 3 Munc13 specifically reduces DCV release at synapses. (A) Average number of DCV release events per cell for WT and Munc13-1/2 DKO (M13DKO) neurons measured with Sema3a-pHluorin (WT: 482 events, n = 41 cells; M13 DKO: 108 events, n = 23 cells, N = 4, ** p < 0.01). (B) Cumulative number of release events per cell. (C) Cumulative number of release events during the first four bursts of 50 AP at 50 Hz. (D) Percentage of synaptically localized DCVs that remain synaptic during 10 s acquisition (WT: 221 vesicles, n = 6 cells; M13 DKO: 146 vesicles, n = 5 cells, N = 2). (E) Percentage of synaptic and extra-synaptic DCV release events in WT and Munc13 DKO neurons (WT: 21 cells and M13 DKO: 23 cells, N = 4, ** p < 0.01).(F) Total numbers of synaptic and extra-synaptic events show a specific loss of synaptic events (total events WT: 255; M13 DKO: 151).(G) Cumulative number of synaptic DCV release events. (H) Cumulative number of extra-synaptic DCV release events.

Hence, Munc13 deletion does not alter DCV localization or mobility at steady state. It does however specifically affect the amount and rate of synaptic DCV release.

Munc13-1 overexpression increases the number of extra-synaptic DCV release events

Munc13-1 overexpression in bovine chromaffin cells enhances DCV release (Ashery et al., 2000). To test if similar principles apply to DCV release in neurons, we tested the effect of Munc13-1 overexpression in WT hippocampal neurons. Munc13-1 overexpression (M13OE) resulted in a more than 4-fold increase in protein levels compared to endogenous levels (Fig. 4A). Endogenous Munc13-1 showed a clear punctate localization, which overlapped with synapsin-mCherry. In contrast, overexpressed Munc13-1 was not restricted to synapses, but distributed homogenously throughout the neurites (Fig. 4B). Similar ectopic expression of Munc13-1 has been observed before (Deng et al., 2011).

Munc13-1 overexpression did not change the total number or localization of DCVs (Fig. S2B, C and Fig. 4C), but significantly increased DCV release events compared to WT (WT: 10.1 ± 1.8, n = 36; M13OE: 18.4 ± 3.2, n = 32, $p < 0.05$, Fig. 4D). Release started earlier in M13OE neurons (Fig. 4E-F), although this effect was rather subtle when measured regardless of the spatial localization of the events. However, as in Munc13 DKO neurons, the preference for DCV release at synaptic sites was lost in M13OE neurons (Fig. 4G). In contrast to Munc13 deletion, this was now caused by a specific increase in DCV release events at extra-synaptic sites (Fig. 4H) with significantly increased vesicle release rates at these sites (WT extra-synaptic: 0.24 ± 0.04 vesicles/sec; M13OE extra-synaptic: 0.36 ± 0.01 vesicles/s, $p < 0.05$, Fig. 4I). In M13OE neurons, extra-synaptic release events occurred already during the first four stimuli. During this time window WT neurons did not show release (Fig. 4J). Munc13 overexpression hardly, if at all, affected synaptic release events (Fig. 4K-L). Together, this shows that overexpression of Munc13-1 is sufficient to produce efficient DCV release at extra-synaptic sites.

A model for DCV release in neurons

The present study characterized mechanisms that control synaptic DCV release in hippocampal neurons. We found that although DCVs are not enriched in synaptic terminals, they preferentially fuse at synapses. Release at extra-synaptic sites only occurs upon more prolonged stimulation compared to synaptic DCV release. We provide evidence that the synaptic priming proteins Munc13-1 and Munc13-2 are, unlike in synaptic vesicle secretion (Varoqueaux et al., 2002), not essential for DCV release, but primarily control the localization and efficiency of DCV release. In their absence, synaptic preference of DCV fusion is lost and remaining release events require stimulation intensities similar to extra-synaptic release events in wildtype neurons, while over-expression promotes extra-synaptic release events that do not require prolonged stimulation.

Co-localization with synapsin-mCherry and post-hoc confirmation using immuno-detection indicated that only a small proportion (± 20 %) of the 100-150 DCV (-clusters) present in WT, M13DKO and M13OE neurons localizes to synaptic sites. Random sections of presynaptic profiles contained on average 1.4 DCV with few postsynaptic DCVs and almost half of the profiles were devoid of DCVs. Hence, our data suggest that there is no strong accumulation of DCVs in hippocampal synapses. Based on the observed DCV distribution, we expected ± 20 % of the DCV release events to occur at synaptic sites. Yet, we found that more than 65 % of all events did. Release at synapses started earlier upon stimulation and reached higher release rates than extra-synaptic release. As we did not find evidence for synaptic accumulation of

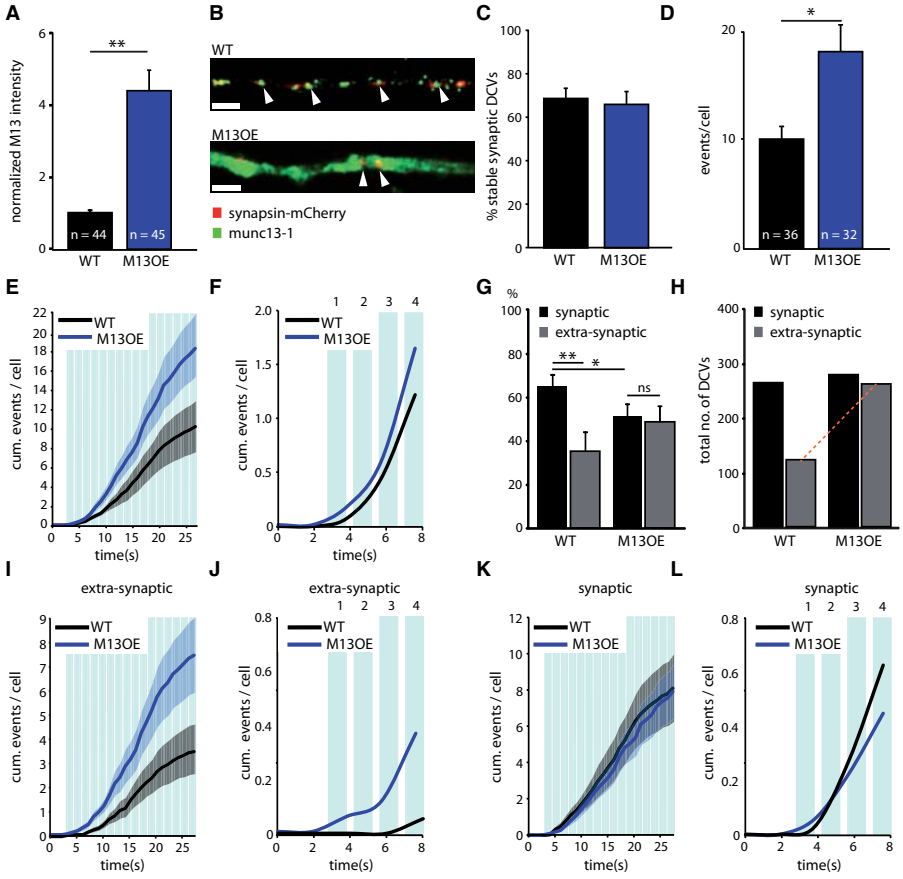

Figure 4 Munc13-1 overexpression increases DCV release at extra-synaptic sites (A) Normalized Munc13-1 intensity in WT neurons compared to neurons overexpressing Munc13-1 (M13OE, N = 3, ** p<0.001). (B) Confocal image of a WT neuron with endogenous Munc13-1 and a WT neuron overexpressing Munc13-1 (arrowheads show synaptic localization of Munc13, scale bars, 2 µm). (C) Percentage of synaptically localized DCVs that stay synaptic for 10 s (WT: 199 vesicles, n = 7 cells; M13OE: 170 vesicles, n = 7 cells). (D) Average number of DCV release events per cell (WT: 361 events in 36 cells; M13OE: 588 events in 32 cells, N = 3, * p < 0.05). (E) Cumulative DCV release events per cell. (F) Cumulative number of release events during the first four bursts of 50 AP at 50 Hz. (G) Percentage of synaptic and extra-synaptic DCV release events (** p < 0.01, * p < 0.05). (H) Total numbers of synaptic and extra-synaptic DCV release events. Loss of synaptic preference is due to an increase of extra-synaptic release events in M13OE. (I) Cumulative extra-synaptic DCV release events. (J) Cumulative number of events during the first four bursts at extra-synaptic sites. (K) Cumulative synaptic DCV release events. (L) Cumulative number of events during the first four bursts at synapses.

DCVs prior to release, we conclude that DCV release probability at synaptic terminals is higher than at extra-synaptic sites. As most DCVs in mammalian neurons are mobile (Ramamoorthy et al., 2011; Silverman et al., 2005) and on average only one in 5 synapses harbors stationary DCVs (Fig 2 and de Wit et al., 2006; Matsuda et al., 2009) which are often not pre-docked (de Wit et al., 2006; de Wit et al., 2009b; Matsuda et al., 2009; Silverman et al., 2005; Verhage et al., 1991),

suggesting that DCVs are recruited to synapses during activity and consequently, dock and fuse from an undocked, mobile state during stimulation.

We found that loss of Munc13-1/2 strongly reduced DCV release and that Munc13-1 is the dominant isoform supporting DCV release. Hence, Munc13 emerges as a general priming factor for regulated secretion of different types of secretory vesicles (synaptic vesicles and DCVs). However, almost 40 % of the fusion events remained in M13DKO neurons, while synaptic vesicle fusion is abolished completely in such neurons (Varoqueaux et al., 2002). In fact, our data show that impaired release efficiency is the main effect of Munc13-1/2 loss: in their absence, the first fusion events occur only after 3-4 episodes of 50 APs at 50 Hz. This is the same type of drastic stimulation, which may be very rare *in vivo*, required to trigger extra-synaptic DCV fusion in wild type neurons. So, DCV fusion appears to lose its synaptic advantage upon Munc13 loss. This is consistent with the highly localized distribution of Munc13-1/2 to active zones (Kalla et al., 2006).

Conversely, Munc13-1 overexpression leads to many more DCV fusion events at extra-synaptic sites, which also required less intense stimulation. Upon overexpression, non-synaptic DCV fusion events appeared to gain synaptic-like properties and overexpression of Munc13-1 appears to be sufficient to specify extra-synaptic DCV release sites. Munc13 interacts with the tSNARE protein syntaxin and the S/M protein Munc18 (Ma et al., 2011; Richmond et al., 2001). These proteins serve as essential and minimal components for membrane fusion in many systems (Rizo and Sudhof, 2002; Toonen and Verhage, 2007; Verhage et al., 2000), and are not exclusively present at synapses (Galli et al., 1995). Munc13 could organize/activate extra-synaptic tSNARE/Munc18 complexes to increase DCV release probability at these sites.

Online supplemental material

Fig. S1 shows DCV release events in wild type neurons measured with NPY-pHluorin, calcium influx upon a stimulation paradigm of 16 bursts of 50 action potentials at 50 Hz, comparison of the effectiveness of different stimulation paradigms for DCV release, co-localization of synapsin-mCherry with endogenous VAMP2 and the number of synapses per neurite length in our culture system. Fig. S2 shows the total number of DCVs present in all genotypes with their distribution. Fig. S3 shows DCV release and EM localization in *munc13-1* null mutant neurons.

Supplemental Figure 1 (A) Normalized cumulative frequency plot of DCV release measured with NPY-pHluorin in WT cells (left y-axis, black line, 159 release events in 16 cells, N = 3), together with the frequency distribution of the release events (right y-axis, red bars). (B) Percentage of DCV release events 30 s before, during and 90 s after the stimulation period (** $p < 0.01$), shows that DCV release is strongly coupled to calcium influx during the stimulation period. (C) Percentage of synaptic and extra-synaptic DCV release events in WT cells (56 synaptic release events, 15 extra-synaptic release events, n = 5, **$p < 0.01$). Shows that DCVs are preferentially released from synaptic regions. (D) Average calcium response measured with Fluo4 upon electrical field stimulation (16 bursts of 50 AP at 50 Hz, n = 56 synaptic regions in 4 cells). (E) Number of release events in response to 16 bursts of 50 AP at 50 Hz compared to other stimulation paradigms (1 AP, 40 AP at 20 Hz, 5 x 40 AP at 100 Hz, n = 5 cells for each condition). (F) Confocal images of a WT neuron transfected with synapsin-mCherry and post-hoc stained for endogenous VAMP2 and MAP2. Synapsin-mCherry strongly overlaps with VAMP2. Scale bar, 10 µm. (G) Zoom of boxed area in (F). Scale bar, 5 µm. (H) Typical example of a hippocampal dendrite stained for VAMP and MAP2. Scale bar, 20 µm. (I) Histogram of synapse density per µm dendrite (130 cells, 559 ± 28 synapses per cell, 1729.0 ± 83.1 µm dendrite per cell analyzed, 5 independent experiments). With ± 0.3 synapsin-labeled terminals per µm dendrite and an average synapse size of 1 µm, the ± 20 % overlap we and others (Matsuda et al., 2009) observed suggests that the overlap between synapses and DCVs is random. ►

ACKNOWLEDGEMENTS

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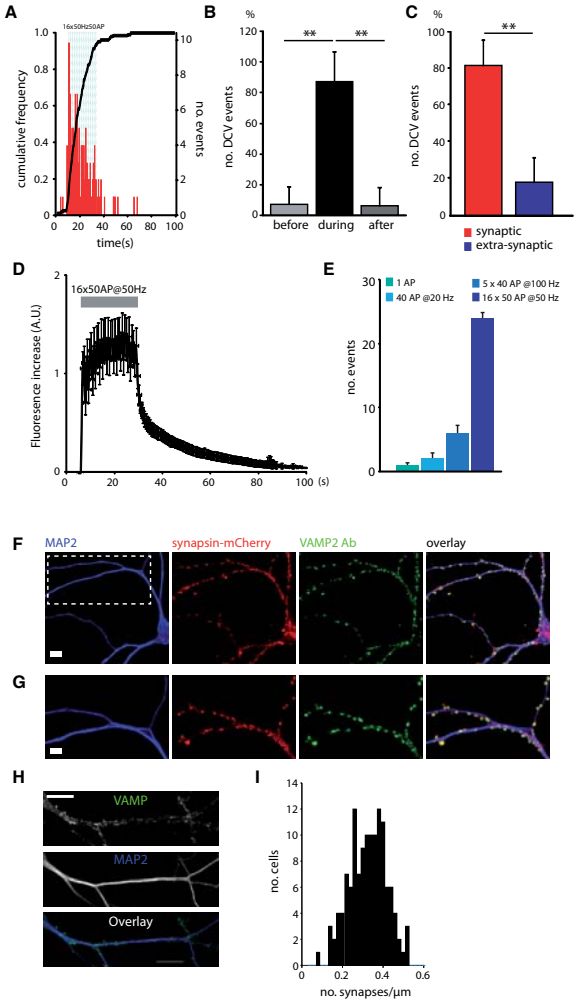
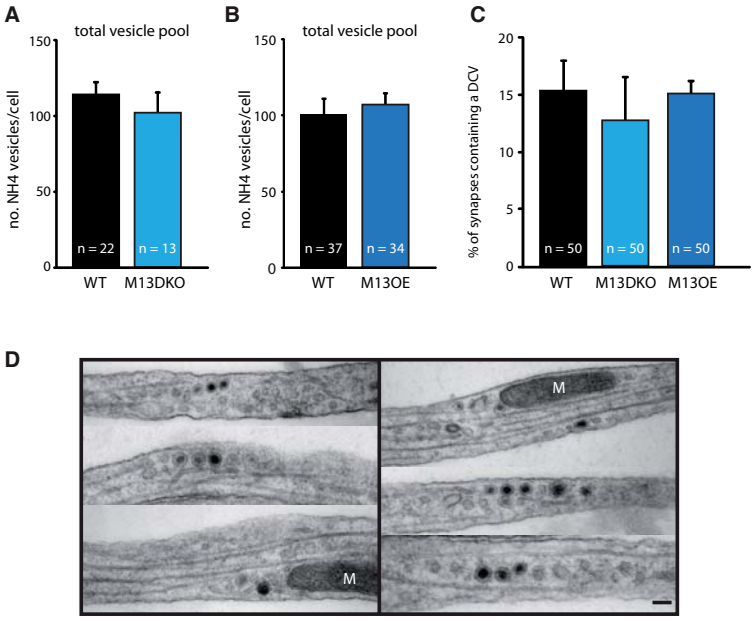
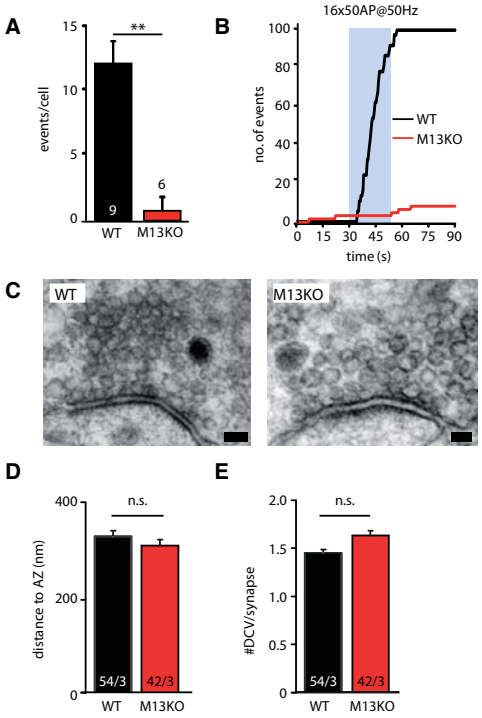
SUPPLEMENTARY FIGURES

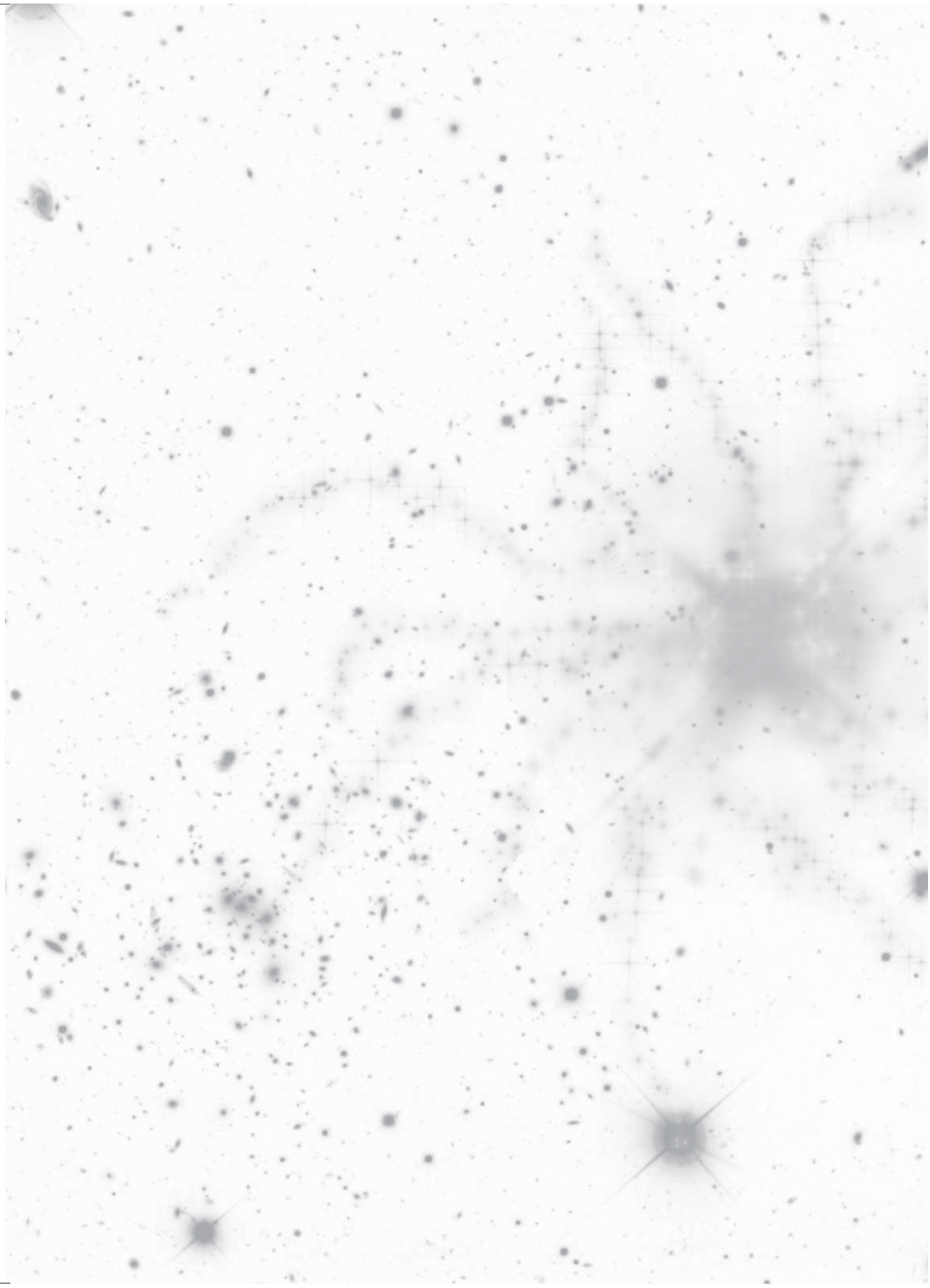




Supplemental Figure 2 (A) Average number of DCVs per neuron upon NH_4+ superfusion to instantly dequench all pHluorin molecules and visualize all DCVs. The total number of DCVs per neuron is similar between Munc13 DKO and WT neurons (WT: 112 ± 8.4 vesicles/cell, n = 22; M13 DKO: 102 ± 10.1 vesicles/cell, n = 13). (B) Average number of DCVs per neuron upon NH_4+ superfusion to instantly dequench all pHluorin molecules. The total number of DCVs per neuron is similar between Munc13 overexpressing (M13 OE) and WT neurons (WT: 101 ± 7.9 vesicles/cell, n = 37; M13 OE: 114 ± 6.1 vesicles/cell, n = 34). (C) Average percentage of synapses containing DCVs measured upon NH_4+ superfusion to instantly dequench all pHluorin molecules and visualize all DCVs. Synapses were identified by synapsin-mCherry co-transfection. In each neuron, 50 random synapses were selected and assessed for co-localization with DCVs. The number of synapses with DCVs does not differ between WT, Munc13 DKO and Munc13 OE neurons (WT: 15 ± 3%, n = 50 synapses in 4 cells; M13DKO: 13.4 ± 5%, n = 50 synapses in 5 cells; M13 OE: 14.9 ± 2%, n = 50 synapses in 4 cells). (D) Representative electron micrographs of DCVs in neurites of WT neurons. DCVs do appear as single vesicles but are also frequently clustered in rows of 2 to 5 vesicles. Note that when clustered, DCVs appear to be aligned to tubulin tracks. M = mitochondrion, calibration bar, 100 nm.



Supplemental Figure 3 (A) Average number of DCV release events per cell for WT and *munc13-1* null mutant (M13KO) neurons measured with NPY-pHluorin (WT: 108 events, n = 9 cells; M13KO: 9 events, n = 6 cells, N = 2, ** $p < 0.01$). (B) Cumulative number of release events during the total acquisition time. Blue bar represents 16 bursts of 50 AP at 50 Hz stimulation. (C) Electron micrographs of synaptic terminals from wildtype (WT) and *munc13-1* null mutant (M13KO) neurons. Bar, 100 nm. (D) Average distance of DCVs present in the presynaptic terminal to the active zone (AZ) is similar between WT and M13 KO neurons (WT: 317 ± 13 nm, n = 54 synapses from 3 independent cultures; M13KO: 302 ± 15 nm, n = 42 synapses from 3 independent cultures). (E) Average number of DCVs per synaptic profile is similar between WT and M13 KO neurons (WT: 1.46 ± 0.04, n = 54 synapses from 3 independent cultures; M13KO: 1.61 ± 0.03, n = 42 synapses from 3 independent cultures).



CHAPTER 3

CAPS PROTEINS CONTROL DCV RELEASE IN HIPPOCAMPAL NEURONS

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In preparation

ABSTRACT

Dense core vesicle (DCV) release of neuropeptides plays an important role in modulating neuronal activity. Priming is an important step preceding vesicle fusion. The active zone proteins αRIM, Munc13 and CAPS are major priming proteins for synaptic vesicle release but it is unclear how priming of DCVs is regulated in neurons. Here, we studied the role of CAPS-1 and CAPS-2 in neuronal DCV release by imaging the release of single DCVs. We identify CAPS-1/2 as important DCV priming proteins at synaptic and extra-synaptic sites. CAPS proteins did not affect the biogenesis or loading of neuronal DCVs. Hence, CAPS-1/2 emerge as important secretory vesicle priming proteins for synaptic vesicle and DCV release in mammalian neurons.

INTRODUCTION

Neuropeptide release from neuronal dense core vesicles (DCVs) modulates many important pathways in the brain. DCV fusion is triggered by calcium influx and requires a biochemical priming step before docked vesicles can fuse with the target membrane. Synaptic vesicle (SV) priming is regulated by family members of the active zone proteins αRIM (Deng et al., 2011), Munc13 (Augustin et al., 1999; Varoqueaux et al., 2002) and calcium-dependent activator protein for secretion (CAPS) (Jockusch et al., 2007). Deletion of either of these proteins strongly affects or totally abolishes synaptic vesicle release (Augustin et al., 1999; Jockusch et al., 2007; Schoch et al., 2002; Varoqueaux et al., 2002).

Munc13-1/2 and CAPS-1/2 share similar protein domains and are both priming factors, but they are unable to replace each other and therefore likely function in a serial pathway each possessing unique SV priming features (Jockusch et al., 2007). CAPS-1 and CAPS-2 have 78% sequence identity and are widely expressed in the brain, both during development and in the adult brain with distinct as well as overlapping expression in neurons of the hippocampus (Speidel et al., 2003). CAPS-1 deletion reduces evoked excitatory postsynaptic currents (EPSCs) and spontaneous release (mEPSC) size and frequency in hippocampal neurons, while CAPS-2 null mutant neurons behave like wild-type littermates (Jockusch et al., 2007). CAPS-1/2 double null mutants show a slightly stronger reduction in evoked and spontaneous release of SVs than CAPS-1 single KO. Thus CAPS-1 is mainly responsible for supporting calcium triggered SV release (Jockusch et al., 2007).

Besides their essential role in regulating SV release, Munc13 proteins also control neuronal DCV release (Chapter 2 of this thesis). A number of findings also implicate CAPS proteins in DCV secretion. CAPS-2 deletion in cerebellar neurons reduces spontaneous NT-3 release (Sadakata et al., 2004) and overexpression of CAPS-2 in cultured neurons increases BDNF release from axons after 8 min. of potassium stimulation (Shinoda et al., 2011). In PC12 cells, CAPS-2 levels correlate with the capacity to release BDNF (Khodthong et al., 2011; Sadakata et al., 2004) and in bovine chromaffin cells, deletion of CAPS-1 and CAPS-2 completely abolishes DCV release (Liu et al., 2010). CAPS proteins are also implicated in catecholamine uptake in secretory vesicles in chromaffin cells (Speidel et al., 2005). Finally, studies in *Drosophila* and *C. elegans* suggest that the CAPS ortholog UNC-31/dCAPS regulates DCV release (Hammarlund et al., 2008; Renden et al., 2001; Weimer et al., 2006). Close examination of *C. elegans* null mutations using electron microscopy suggests that in addition to the role in DCV priming, UNC-31 also functions in DCV docking. However, how the two CAPS isoforms regulate activity-dependent DCV release from mammalian neurons is not known.

In this study we investigated DCV release and localization in CAPS-1/2 knockout mice. CAPS deletion did not affect the loading of DCVs with peptidergic cargo but led to a severe reduction of DCV release without changing the subcellular location of DCV release. Both in WT and CAPS-1/2 DKO neurons, DCVs were preferentially released from synaptic regions. The release rates of DCVs in CAPS-1/2 knockout neurons were strongly reduced at both synaptic and extra-synaptic sites. Hence, CAPS proteins are important priming factors for neuronal DCV release that facilitate DCV fusion irrespective of their spatial distribution.

RESULTS

CAPS deletion does not influence DCV loading in hippocampal neurons

CAPS proteins are implicated in catecholamine uptake into secretory vesicles in chromaffin cells (Speidel et al., 2005). As a defect in vesicle loading could influence our analysis of release events, we first tested for potential effects of CAPS deletion on neuronal DCV loading with neuropeptides. The fluorescent intensity of the released events of WT and CAPS-1/2 DKO showed a similar distribution, showing that vesicle loading of these released DCVs is not affected in CAPS-1/2 DKO neurons (Fig 1A). For analyzing the fluorescent intensity of all DCVs in the cell, we used two semi-quantitative measurements of fluorescent intensity of pHluorin labeled DCVs upon application of NH_4^+ (to instantly de-quench all intra-vesicular pHluorin, Fig 1B) and of antibody-labeled endogenous DCV cargo, secretogranin II, in WT and CAPS-1/2 DKO neurons. NH_4^+ application showed that loading of DCV-pHluorin is comparable between WT and CAPS-1/2 DKO neurons (Fig 1C-D). Also, fluorescent intensity levels of secretogranin II were unchanged between WT and CAPS-1/2 DKO neurons, suggesting that neuropeptide content of DCVs is not affected by deletion of CAPS-1/2 (Fig 1E). In addition, CAPS-1/2 deletion did not affect vesicle biogenesis or neuronal morphology (Fig 1F-H).

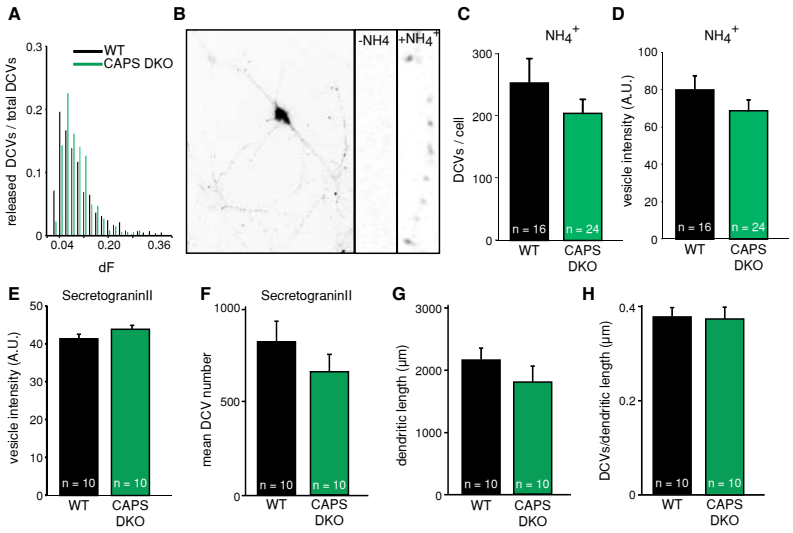


Figure 1 CAPS deletion does not affect neuropeptide loading of DCVs. (A) Distribution of the fluorescent intensity increase (dF) of the released DCVs in WT and CAPS-1/2 DKO neurons. The number of DCVs per bin is corrected for the total number of DCVs released. (B) Inverted wide-field image of a neuron expressing cargo-pHluorin upon NH_4+ application to reveal all DCVs present in the cell. Zoom shows the effect of vesicle de-acidification upon NH_4+ application (-NH_4 before application, +NH_4 during application). (C) Average number of DCVs per neuron, quantified from the NH_4+ response in WT and CAPS-1/2 DKO neurons. (D) Average intensity of single DCVs quantified from the NH_4+ response in WT and CAPS-1/2 DKO neurons (WT 4435 vesicles, CAPS DKO 4892 vesicles). (E) Average intensity of individual DCVs in the field of view of confocal images of non-transfected wild-type (WT) or CAPS-1/2 DKO neurons (CAPS DKO) neurons stained for the endogenous DCV cargo secretogranin II and dendrite marker MAP2 (n = 20 cells). (F) Average number of DCVs per field of view. (G) Average dendritic length per field of view. (H) Number of DCVs per dendritic length.

Deletion of CAPS proteins results in slower and reduced DCV release

To understand the function of CAPS-1 and CAPS-2 in neuronal DCV release, we compared CAPS-1/2 DKO, and CAPS-1 and CAPS-2 single null mutants with wild-type (WT) hippocampal neurons. DCV release was triggered by electrical stimulation using a protocol known to elicit robust BDNF release from neurons (16 bursts of 50 AP at 50 Hz, Hartmann et al., 2001). CAPS-1/2 DKO neurons showed a more than 50% reduction in the number of DCVs released during stimulation compared to WT (Fig 2A, WT 23.7 ± 4.5 events/cell, n = 24; CAPS DKO 9.6 ± 1.5 events/cell, n = 32, ** p < 0.01). The average vesicle release rates per cell during stimulation, calculated from the cumulative release plots (Fig 2B) were 1.2 ± 0.4 vesicles/s for WT compared to 0.15 ± 0.1 vesicles/s for CAPS-1/2 DKO neurons. DCV release in single CAPS-1 KO and CAPS-2 KO neurons was not significantly different from WT (CAPS-1 KO 14.5 ± 3.9 events/cell, n = 19; CAPS-2 KO 14.8 ± 3.0 events/cell, n = 33, p = 0.14 for WT vs. CAPS-1 KO and p = 0.07 for WT vs. CAPS-2 KO, Fig 2A-B).

As priming defects become most evident at the start of stimulation, we zoomed in on the first four bursts of the stimulation paradigm. The number of release events during this period was significantly lower in CAPS-1 and CAPS-1/2 DKO neurons, while release in CAPS-2 KO neurons was similar to WT (WT 4.8 ± 1.5 events/cell, n = 24; CAPS-1 2.1 ± 1.0 events/cell, n = 19; CAPS DKO 1.4 ± 0.3 events/cell, n = 32, CAPS-2 KO 4.3 ± 1.2 events/cell, n = 33; * p < 0.05, Fig 2C).

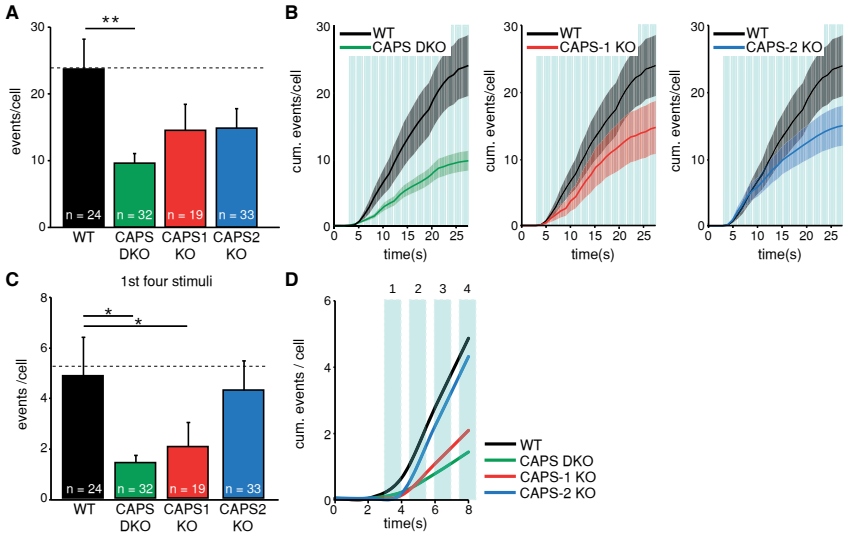


Figure 2 Deletion of CAPS proteins results in delayed and reduced DCV release. (A) DCV release events for WT, CAPS-1 KO, CAPS-2 KO and CAPS-1/2 DKO neurons during stimulation (WT total 569 events, 23.7 ± 4.5 events/cell, n = 24; CAPS-1 KO total 276 events, 14.5 ± 3.9 events/cell, n = 19; CAPS-2 KO total 489 events, 14.8 ± 3.0 events/cell, n = 33; CAPS DKO total 307 events, 9.6 ± 1.5 events/cell, n = 32; N = 4 independent experiments for all groups except CAPS-1 KO (N = 2), ** p<0.01). (B) The average cumulative frequency of DCV release events per cell during stimulation in WT compared to CAPS KO neurons (blue bars represent 16 bursts of 50 AP at 50 Hz, error bars represent SEM). (C) Average number of release events for the first four bursts for WT and CAPS KO cells (WT vs. CAPS DKO, * p < 0.05; WT vs. CAPS-1 KO, * p < 0.05, WT vs. CAPS-2 KO n.s.). (D) Zoom of the cumulative frequency of DCV release during the first four bursts of stimulation (blue bars 1, 2, 3, 4 each represent 50 AP at 50 Hz) for WT, CAPS-1 KO, CAPS-2 KO and CAPS-1/2 DKO.

To measure how fast DCVs are released upon stimulation, we analyzed the time of onset of each individual DCV release event during the first four stimulation bursts. Release in CAPS-1 KO and CAPS-1/2 DKO neurons was delayed compared to WT and CAPS-2 KO neurons with release occurring only after the second burst of stimulation (Fig 2D). Together, these findings show that deletion of both CAPS isoforms strongly affects activity-dependent release of neuronal DCVs by reducing the total amount of secreted vesicles and delaying the onset of DCV release upon stimulation. Deletion of CAPS-1 mimics the CAPS-1/2 DKO phenotype, especially during the first four stimulation bursts. Deletion of CAPS-2 appears to affect vesicle release rates only after repeated stimulations (Fig 2B).

CAPS deletion affects DCV release at synaptic and extra-synaptic sites

CAPS proteins are enriched at presynaptic sites (Renden et al., 2001; Speidel et al., 2003). To test if CAPS deletion specifically affects release at synaptic sites, we investigated DCV release from neurons transfected with the synaptic vesicle protein synapsin fused to mCherry. Synapsin-mCherry specifically labels presynaptic terminals (Chapter 2) and allows the categorization of release events into synaptic and extra-synaptic events. In line with our observations described in Chapter 2, WT cells showed a clear preference for synaptic release events (Fig 3A). Deletion of CAPS-1/2 affected synaptic and extra-synaptic release to a similar extent and therefore did not change the synaptic preference of DCV release (Fig 3A). The total number of DCV release events for WT and CAPS-1/2 DKO neurons in this subset of the cells shown in Figure 2 was slightly higher, but release in CAPS 1/2 neurons was still ± 50% of WT (synaptic: WT 18.1 ± 3.5, n = 20; CAPS DKO 9.0 ± 2.5, n = 28, *p < 0.05; extra-synaptic: WT 8.9 ± 1.8, n = 20; CAPS DKO 4.9 ± 0.9, n = 28, p = 0.09, Fig 3B). DCV release rates in CAPS-1/2 DKO neurons, obtained from linear fits of the cumulative release plots per cell, were reduced at both synaptic and extra-synaptic sites (synaptic WT 0.93 ± 0.4 vesicles/s, CAPS DKO 0.48 ± 0.2 vesicles/s, extra-synaptic WT 0.46 ± 0.2 vesicles/s, CAPS DKO 0.1 ± 0.2 vesicles/s, Fig 3C-D). The delayed onset of release in the CAPS-1 KO in the first four pulses of release was evident both at synaptic and extra-synaptic sites (Fig 3E-F). Thus, CAPS deletion strongly affects DCV release but does not specifically reduce DCV release at synaptic locations.

DISCUSSION

In this study we showed that CAPS proteins are major determinants of DCV release from mammalian neurons. CAPS deletion did not affect vesicle loading with neuropeptides as previously reported for catecholamine loading of DCVs in CAPS-1 deficient chromaffin cells (Speidel et al., 2005). In CAPS-1/2 DKO neurons, DCV release was reduced by more than 50 %. Despite the fact that CAPS proteins are enriched in synapses, DCV release was equally affected at both synaptic and extra-synaptic sites. Hence, CAPS proteins function as important priming factors for neuronal DCV release but do not provide spatial specificity.

Our data implicate CAPS proteins as important priming factors in DCV release. In SV release, deletion of both CAPS isoforms also results in a strong reduction of spontaneous and evoked release (Jockusch et al., 2007). Deletion of both isoforms in chromaffin cells showed an even stronger phenotype with a total arrest of evoked release (Liu et al., 2010). Hence, CAPS-1/2 proteins are important priming factors for release of synaptic vesicles and are essential for release of DCVs from adrenal chromaffin cells. In contrast to the latter observation, we found

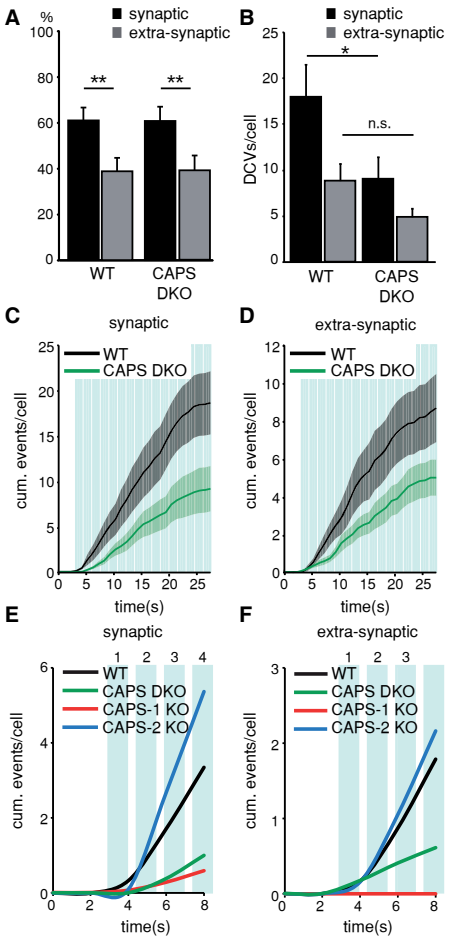


Figure 3 CAPS deletion reduces DCV release rates at synaptic and extra-synaptic sites. (A) Percentage of synaptic and extra-synaptic DCV release events (** $p < 0.01$). CAPS deletion does not change this ratio. (B) Average number of DCVs released per cell at synaptic sites (synaptic: WT 18.1 ± 3.5, n = 20; CAPS DKO 9.0 ± 2.5, n = 28, $p < 0.05$) and extra-synaptic sites (extra-synaptic: WT 8.9 ± 1.8, n = 20; CAPS DKO 4.9 ± 0.9, n = 28, p = 0.09) for WT and CAPS-1/2 DKO neurons. (C) Average cumulative frequency of synaptic DCV release events per cell in CAPS-1/2 DKO neurons compared to WT neurons during stimulation (error bars represent SEM, WT 19 cells, CAPS DKO 28 cells). (D) Average cumulative frequency of extra-synaptic DCV release events per cell in CAPS-1/2 DKO neurons compared to WT neurons during the entire acquisition period (error bars represent SEM). (E) Zoom of the cumulative frequency of synaptic DCV release during the first four bursts of stimulation (blue bars 1, 2, 3, 4 each represent 50 AP at 50 Hz) for WT, CAPS-1 KO, CAPS-2 KO and CAPS-1/2 DKO. (F) Zoom of the cumulative frequency of extra-synaptic DCV release during the first four bursts of stimulation.

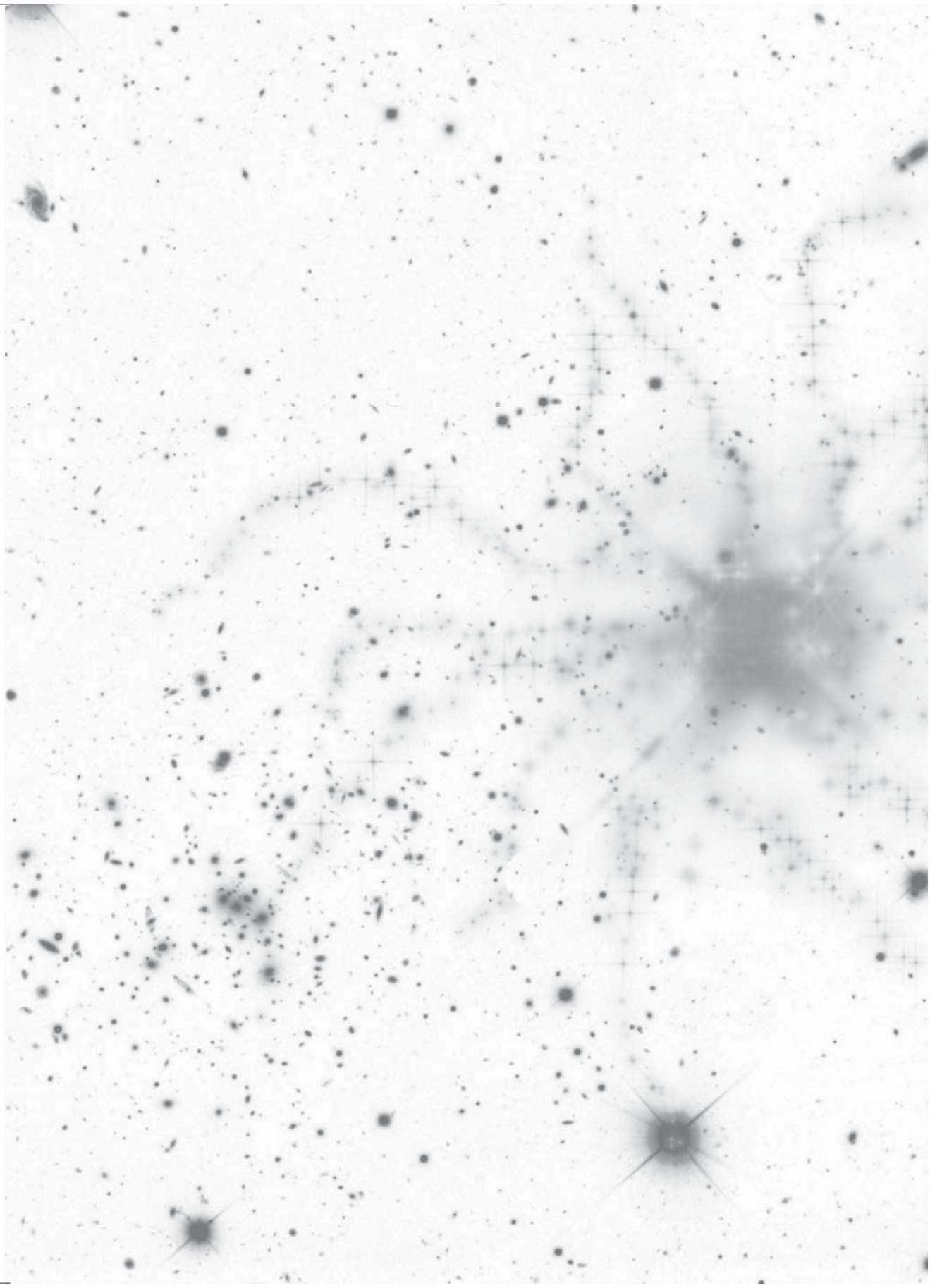
that in hippocampal neurons deletion of both CAPS-1 and CAPS-2 resulted in decreased DCV release but it did not abolish DCV release. This, combined with the results from deletion of Munc13-1/2 described in Chapter 2, shows that neuronal DCV release does not require the presence of two major vesicle priming factors and suggests that neuronal DCV release may use different release machinery than two of the most studied secretion assays, synaptic vesicle release and DCV release from chromaffin cells.

Deletion of either Munc13-1/2 or CAPS-1/2 resulted in a similarly strong reduction of DCV release. However, where Munc13 specifically affects release at synapses (see Chapter 2), we show here that CAPS regulates DCV release at synaptic and extra-synaptic sites. Both proteins thus function as vesicle priming factors but they may function in vesicle priming in completely different ways. The fact that both proteins have unique functions in DCV release is also supported by the observation that they have a differential function in SV release. Munc13 overexpression cannot rescue SV release defects in CAPS-1/2 DKO neurons and vice versa (Jockusch et al, 2007). Although CAPS and Munc13 both contain a so-called Munc13 homology

domain (MUN domain), which is important for their function in synaptic vesicle priming, they also have a number of specific domains that might explain the difference in capacity to affect synaptic release. CAPS proteins contain a DCV binding domain at their C-terminus, which is required for its function and a PIP2 binding PH-domain at the N-terminus. CAPS proteins may function to bridge the gap between DCVs and PIP2 containing plasma membranes (Grishanin et al., 2002). Hence, CAPS proteins may be present on transported DCVs and could be sufficient to drive fusion of DCVs upon calcium influx. Removal of CAPS in our CAPS-1/2 mutant mice indeed affected release efficiency regardless of spatial location. This supports a model in which CAPS proteins present on the DCV membrane are important to promote DCV release irrespective of their spatial location and that the accumulation of CAPS proteins in synaptic terminals mainly serves to support release of synaptic vesicles.

We did not find evidence for a defect in vesicle loading in CAPS KO neurons. Both exogenous and endogenous neuropeptide content was similar between WT and CAPS null mutants. In line with our observation, knockdown of CAPS-1 with shRNAs in PC12 cells also does not change neuropeptide loading in secretory vesicles (Fujita et al., 2007). The fact that CAPS-1 does influence catecholamine loading in chromaffin cells may suggest that the mechanism for loading DCVs with neuropeptides or catecholamines in these different cell types is regulated differently. Transport of monoamines into secretory vesicles is mediated by VMATs (vesicular monoamine transporters). The two isoforms of VMAT are differentially expressed in mammals; VMAT1 is restricted to neuro-endocrine cells while VMAT2 is the only isoform expressed in neurons (Erickson et al., 1992; Liu et al., 1992). Although unlikely, it might be that VMATs function in a CAPS-1 dependent manner. In contrast to monoamine loading, neuropeptides are loaded into secretory vesicles in the Golgi, while VMATs are part of the vesicular membrane and therefore loading is not restricted to specific subcellular locations like the Golgi.

Both CAPS proteins are involved in regulating DCV release. However, during the first three stimulation bursts, CAPS-1 deletion appeared to affect DCV release much more than deletion of CAPS-2, which showed DCV release similar to WT cells. The vesicles that fuse during the first four stimulation bursts are the most release ready DCVs, suggesting that CAPS-1 is the major CAPS isoform involved in controlling the release readiness of DCVs in hippocampal neurons. However, we cannot rule out that differences in expression levels of CAPS-1 and -2 in the hippocampus may contribute to this phenotype. For instance, CAPS-2 has been implicated in the regulation of spontaneous DCV release from cerebellar neurons (Sadakata et al., 2004), where CAPS-2 expression is much higher than CAPS-1 expression (Speidel et al., 2003). In the hippocampus, both isoforms are expressed in a roughly complementary fashion with high expression of CAPS-1 mainly in CA3 and CAPS-2 mainly in CA1 while dentate gyrus cells express both genes at more moderate levels (Sadakata et al., 2006). Sub-region specific cultures could be used to address this issue. We conclude that both CAPS isoforms regulate DCV release and that in the hippocampus CAPS-1 is the main isoform controlling DCV release.



CHAPTER 4

ACTIVITY-DEPENDENT DENSE-CORE VESICLE RELEASE IN DEVELOPING NEURONS IS INDEPENDENT OF MUNC18-1

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ABSTRACT

SNARE complexes that connect the target and vesicle membrane are the core machinery to fuse two opposing lipid membranes. In all SNARE dependent membrane fusion processes, SM (Sec1p/Munc18) proteins play an essential role. Synaptic vesicle fusion is completely abolished in the absence of Munc18-1, but which SM protein controls the release of dense core vesicles (DCVs) in neurons is unclear. Here, we investigated DCV release in developing neurons at four days in vitro (DIV4) and show that these neurons contain fewer DCVs than mature neurons and that release is less synchronous. Interestingly, DCV release is not abolished in *munc18-1* null mutant neurons at DIV4, while in older neurons at DIV14 2-fold overexpression of Munc18-1 does not result in increased DCV release. Hence, Munc18-1 is not the major SM protein that controls neuronal DCV release during development and in contrast to release from neuroendocrine cells does not appear to be rate-limiting in DCV release from mature neurons.

INTRODUCTION

Secretory vesicle exocytosis is a highly regulated and conserved process in eukaryotes (see for recent reviews, Jahn, 2000; Sudhof and Rothman, 2009). The release of synaptic vesicles requires the assembly of t-SNARE proteins syntaxin-1 and SNAP25 and the v-SNARE protein Synaptobrevin2 into fusion complexes. In addition, it requires the Sec1p/Munc18 (SM) protein Munc18-1, as deletion of this gene results in a complete loss of synaptic vesicle fusion (Verhage et al., 2000). In addition to their upstream role in vesicle docking (Toonen et al., 2006; Voets et al., 2001), SM-proteins may function to set up SNARE complexes or may operate as an integral part of assembled fusion complexes (see for recent reviews, Burgoyne and Morgan, 2007; Toonen and Verhage, 2007; Verhage and Toonen, 2007; Sudhof and Rothman, 2009; Carr and Rizo, 2010).

We have recently shown that Botulinum and tetanus toxin sensitive SNARE proteins form the minimal protein machinery necessary to release DCVs in neurons (de Wit et al., 2009b and Chapter 5 of this thesis), but which SM protein functions in neuronal DCV release is not known. Mammals express three Munc18-1 isoforms (Munc18-1, Munc18-2 and Munc18-3) of which Munc18-1 is primarily expressed in the brain and in neuro-endocrine cells (Garcia et al., 1994; Hata et al., 1993; Pevsner et al., 1994). Munc18-2 and Munc18-3 are widely expressed in different tissues, with relatively low expression in brain (Hata and Sudhof, 1995). Munc18-2 is essential for cytotoxic granule exocytosis from natural killer cells and its deficiency causes familial hemophagocytic lymphohistiocytosis type 5 (Cote et al., 2009; Meeths et al., 2010). Munc18-3 is involved in granule release from pancreatic β cells in a complex with syntaxin-4 (Thurmond et al., 2000).

Munc18-1 plays an important role in the release of DCVs from neuro-endocrine cells. In adrenal chromaffin cells, the lack of Munc18-1 strongly hampers DCV docking and release (Voets et al., 2001) and a more recent study shows that Munc18-1 is part of the minimal docking and release machinery (de Wit et al., 2009a). On the other hand, Munc18-1 overexpression in these cells leads to increased DCV docking and release (Voets et al., 2001). In line with these observations, expression of a gain of function mutant in PC12 cells increases the number of DCVs at the plasma membrane (Graham et al., 2008; Voets et al., 2001) and overexpression of a syntaxin-binding deficient Munc18-1 isoform regulates fusion pore dynamics (Fisher et al., 2001) although fusion pore dynamics in *munc18-1* null mutant chromaffin cells are not different from dynamics in wildtype cells (Gulyas-Kovacs et al., 2007). Finally, in pituitary cells, Munc18-1 deletion also results in decreased release of growth hormone and a strong reduction in the number of docked DCVs (Korteweg et al., 2005). Hence, Munc18-1 functions at multiple stages of DCV release in neuro-endocrine chromaffin and pituitary cells. The role of Munc18-1 in DCV release from neurons in the central nervous system however, has not been addressed.

Because of its high expression in brain, Munc18-1 has been considered the most likely candidate to function in neuronal DCV release. We therefore tested the role of Munc18-1 in DCV release from hippocampal neurons. As *munc18-1* null mutant neurons do not survive beyond 5 days in vitro (Heeroma et al., 2004), we examined DCV release from developing, immature neurons at 4 days in vitro (DIV4). We found that DCVs are present in immature neurons albeit at strongly reduced numbers compared to mature neurons at DIV14. We compared DCV release in neurons at DIV4 and DIV14 using an optical DCV release sensor and show that immature neurons release less DCVs upon stimulation than mature neurons. At both developmental stages, DCV release is triggered by the rise of intracellular calcium upon electrical stimulation although relatively more DCVs are released after the stimulus train in immature neurons.

Interestingly, Munc18-1 deletion in immature neurons or a 2-fold overexpression of Munc18-1 in mature neurons does not affect the amount of DCV release nor does it significantly change the vesicle release characteristics. Hence, Munc18-1 does not appear to be the major SM protein that controls neuronal DCV release during development and in contrast to release from neuroendocrine cells does not appear to be rate-limiting in DCV release from mature neurons.

RESULTS

Immature neurons show reduced DCV release compared to mature neurons

We investigated DCV release characteristics in immature (DIV4) and mature (DIV14) neurons to test whether DCV release shows similar temporal and spatial aspects and requires similar fusion molecules during development as in mature neurons. For this, we transfected hippocampal neurons with a DCV cargo protein attached to pH sensitive EGFP (cargo-pHluorin). This allowed us to monitor the number of DCVs released per cell and the timing of these release events. We used brief NH_4^+ application to visualize the total pool of cargo-pHluorin labeled vesicles in the cell. Immature neurons at DIV4 were stimulated with electrical field stimulation of 16 bursts of 50 AP at 50 Hz as described before in Chapter 2 and DCV release was compared to mature DIV14 neurons, which have fully developed synaptic contacts. A histogram of all release events over time shows that immature neurons secreted less DCVs compared to mature neurons (Fig 1A). The number of DCV release events during stimulation in immature neurons was four-fold lower compared to DCV release events in mature neurons (DIV4: 4.2 ± 1.2 events/cell; DIV14: 16.4 ± 3.6 events/cell, ***p<0.001, Fig 1B).

To test whether the lower number of fusion events in immature neurons could be explained by a difference in total vesicle numbers between immature and mature neurons, we counted the total number of vesicles per neuron after NH_4^+ application in a subset of the neurons presented in figure 1A and B. The total number of DCVs in immature neurons was more than 2-fold lower than in mature neurons (DIV4: 173 ± 20.0 vesicles/cell, n=18; DIV14: 399 ± 61.6 vesicles/cell, n = 14, ** p<0.01, Fig 1C). DCV release expressed as percentage of the total vesicle pool was similar between immature and mature neurons (DIV4: 4.8% ± 1.2; DIV14: 6.2% ± 1.6, p = 0.46, Fig 1D). Thus, immature neurons release fewer DCVs upon electrical stimulation and contain less DCVs compared to mature neurons. To test for differences in release rates, we plotted cumulative DCV release events over time, which revealed that the release rate of DCVs in immature neurons was lower during stimulation than in mature neurons (DIV4: 0.2 ± 0.2 vesicles/s, DIV14: 1.0 ± 0.7 vesicles/s, Fig 1E). However, when normalized to the total number of released DCVs, no difference in release kinetics was observed between immature and mature neurons (Fig 1F). This suggests that the lower release rate in immature neurons is due to a smaller release pool rather than a lower vesicle release probability.

DCV release from immature neurons continues after stimulation when intra-cellular calcium levels returned to pre-stimulation levels

As calcium channels are (re-)inserted into the membrane at developmental stage DIV3/DIV4, we tested whether electrical stimulation triggers a similar calcium influx in mature and immature neurons with the calcium indicator Fluo4. Electrical field stimulation (16 bursts of 50 AP at 50 Hz) resulted in a robust increase in global calcium levels at start of the stimulation, which returned

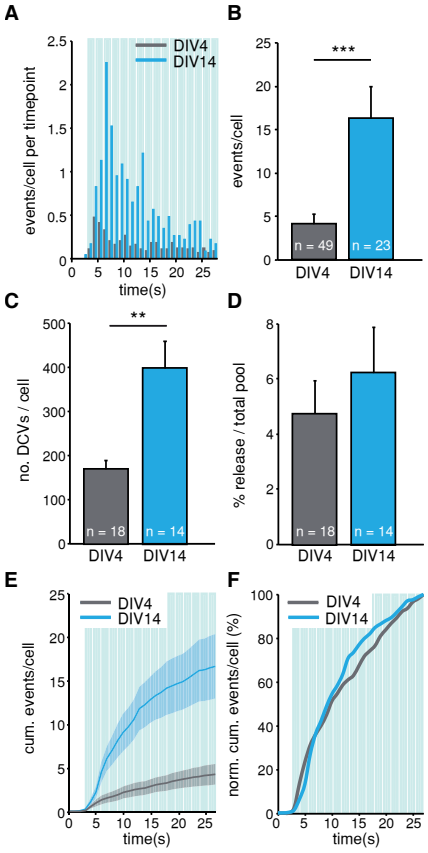


Figure 1 Immature neurons release fewer DCVs upon stimulation than mature neurons. (A) Histogram showing all release events for immature (DIV4) and mature (DIV14) neurons during the 16 bursts of 50 APs at 50 Hz stimulation period (blue bars represent the stimulation of 16 bursts of 50 AP at 50 Hz). (B) Average number of DCV release events during stimulation for immature (DIV4) and mature (DIV14) neurons (DIV4: n = 49 cells, 205 events; DIV14: n = 23 cells, 377 events, N = 6 experiments, Mann-Whitney *** p<0.001). (C) Total number of vesicles per cell upon NH_4 application to visualize all vesicles in a subset of the neurons shown in Figure 1A (DIV4: n = 18 cells, 3106 vesicles; DIV14: n = 14 cells, 5579 vesicles, N = 3 experiments, t-test Welch corrected **p < 0.01). (D) Average percentage of DCVs released of the total number of vesicles per cell (DIV4: 4.8 ± 1.2; DIV14: 6.2 ± 1.6, p = 0.46). (E) Average cumulative frequency of DCV release events during stimulation. (F) Normalized cumulative frequency plot of DCV release events.

to baseline after stimulation (Fig 2A). A similar result was obtained in mature neurons (Fig 2B), showing that calcium influx triggered robust DCV release in both immature and mature neurons. To test whether release arrests after the stimulus train when calcium levels strongly decline, we quantified DCV release during a 60 s time window after stimulation. DCV release in immature neurons was still reduced compared to mature neurons during this time window (DIV4: 1.6 ± 0.3 events/cell; DIV14: 3.3 ± 0.8 events/cell, * p<0.05, Fig 2E). However in immature cells relatively more DCVs were released after the stimulus when calcium levels were back to baseline (Fig 2F).

DCV release is not abolished in *munc18-1* null mutant neurons at DIV4

To investigate whether Munc18-1 is important for DCV release in immature neurons we used *munc18-1* KO neurons and monitored DCV release at DIV3. DCV release in immature *munc18-1* KO neurons was not reduced compared to immature WT neurons (WT: 1.5 ± 0.2 events/cell; M18 KO: 1.1 ± 0.3 events/cell, p = 0.23, Fig 3A-B). The release rate of DCVs in immature *munc18-1* KO neurons during stimulation was comparable with the release rate in WT neurons (WT: 0.09 ± 0.05 vesicles/s; M18 KO: 0.05 ± 0.03 vesicles/s, Fig 3C) with no evidence of a delayed response upon stimulation. Calcium measurements with Fluo4 revealed that calcium influx and efflux dynamics where not significantly different between *munc18-1* KO neurons and WT

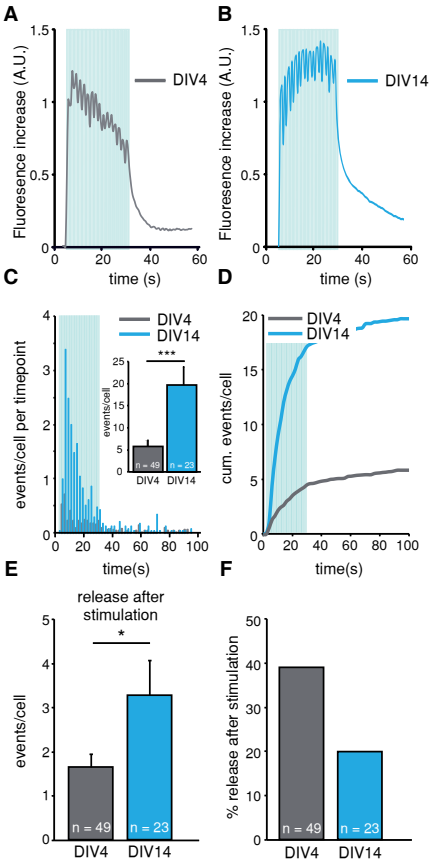


Figure 2 Immature neurons show a similar calcium drop at the end of stimulation compared to mature neurons but continue to release DCVs. (A) Typical example of a calcium trace measured with Fluo4 in neurites of a immature (DIV4) hippocampal neuron in response to 16 bursts of 50 APs at 50 Hz. (B) Calcium trace (Fluo4) in a mature (DIV14) hippocampal neuron in response to 16 bursts of 50 APs at 50 Hz. (C) Histogram showing all release events for immature (DIV4) and mature (DIV14) neurons during the total acquisition time (blue bars represent the stimulation of 16 bursts of 50 APs at 50 Hz). Inset shows the average number of DCV release events during the total duration of imaging (DIV4: n = 49 cells, 285 events; DIV14: n = 23 cells, 452 events, N = 6 independent experiments, Mann-Whitney ***<0.001). (D) Average cumulative frequency of DCV release events during the total acquisition time (blue bars represent the stimulation of 16 bursts of 50 APs at 50 Hz). (E) Average number of DCV release events during a 60 s time period after stimulation for both immature (DIV4) and mature (DIV14) neurons (DIV4: n = 49 cells, 80 events; DIV14: n = 23 cells, 75 events, N = 6 independent experiments, Mann-Whitney *p<0.05). (F) DCV release events after stimulation (Figure 2E) expressed as a percentage of the release events during stimulation (Figure 1B).

neurons (Fig 3D). Munc18-1 deletion also did not change the amount of DCVs released after the stimulation period (WT: 0.9 ± 0.2 vesicles/cell; M18KO: 1.1 ± 0.2 vesicles/cell, p = 0.54, Fig 3E-F). Finally, deletion of Munc18-1 in immature neurons reduced the total number of DCVs per cell (WT: 216.6 ± 28.3 vesicles/cell, M18KO: 96.7 ± 15.4 vesicles/cell, **p < 0.01, Fig 3G) but *munc18-1* KO neurons also showed reduced neurite length (WT: 2179.3 ± 167.6 µm, M18KO: 995.8 ± 185.7, **p<0.01, Fig 3H). Taking the reduced neurite length of the *munc18-1* KO neurons into account resulted in a similar number of DCVs per µm neurite length (WT: 0.11 ± 0.02 vesicles/µm, M18KO: 0.13 ± 0.04 vesicles/µm, p = 0.5456, Fig 3I). Together, these results show that in immature neurons, Munc18-1 is not required for DCV release.

Overexpression of Munc18-1 does not increase DCV release in mature neurons

Synaptic vesicle release and DCV release from adrenal chromaffin cells is markedly increased upon overexpression of Munc18-1 (Toonen et al., 2006; Voets et al., 2001). To test whether DCV release in neurons can also be increased, we overexpressed Munc18-1 in mature neurons and analyzed DCV release numbers and kinetics. Neurons were transfected with wildtype Munc18-1 at DIV10 and analyzed four days later. Overexpression of Munc18-1 resulted in a more than

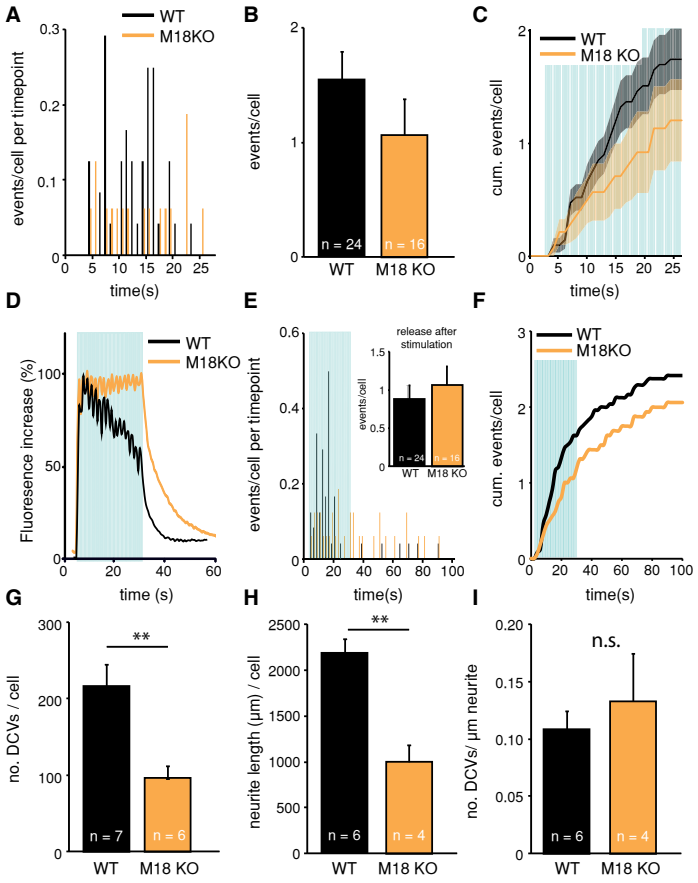


Figure 3 DCV release is not abolished in *munc18-1* null mutant neurons at DIV4. (A) Histogram showing all release events for wildtype (WT) and munc18-1 null mutant neurons (M18KO) neurons during the 16 bursts 50 APs at 50 Hz stimulation period (bin size = 1 sec). (B) Average number of DCV release events during stimulation for WT and M18KO neurons at DIV4 (WT: n = 24 cells, 37 events; M18 KO: n = 16 cells, 17 events, N = 4 experiments, p = 0.23). (C) Cumulative frequency plot of DCV release events in WT and M18KO neurons (blue bars represent the stimulation of 16 bursts of 50 APs at 50 Hz). (D) Typical calcium trace (Fluo4) of a WT and a M18KO neuron at DIV4 (blue bars represent the stimulation of 16 bursts of 50 AP at 50 Hz. (E) Histogram showing all release events for wildtype (WT) and M18KO mutant neurons during the total acquisition time (bin size = 2 sec). Inset shows the average number of DCV release events in a 60 sec time period after the stimulation (WT: n = 24 cells, 21 events; M18KO: n = 16 cells, 17 events). (F) Average cumulative frequency of DCV release events during the total acquisition time (blue bars represent the stimulation of 16 bursts of 50 APs at 50 Hz). (G) Total number of vesicles per cell using NH_4 application to visualize all vesicles (WT: n = 7 cells, 1516 vesicles; M18OE: n = 6 cells, 580 vesicles, **p < 0.01, N = 2 experiments for KO, 1 experiment for WT). (H) Average dendritic length (µm) per cell using SynD to analyze the neurite length from the ECFP image of every cell (WT: n = 6 cells, KO = 4 cells, **p < 0.01, N = 1 experiment). (I) Average number of DCVs per micrometer dendrite length (WT: n = 6 cells, KO = 4 cells, p = 0.55, N = 1 experiment).

2-fold increase in cellular Munc18-1 levels compared to WT cells (WT: 211 ± 8.3 a.u.; M18OE: 527 ± 82.1 a.u., ** $p < 0.01$, Fig 4B). Munc18-1 was distributed throughout the neurites after overexpression (Fig 4A). Munc18-1 overexpression did not change the total number of DCVs per cell (WT: 399 ± 61.6 vesicles/cell; M18OE: 389 ± 31.4 vesicles/cell, Fig 4C). Despite the higher levels of Munc18-1, the number of DCV release events during stimulation was similar between WT and Munc18-1 overexpressing neurons (WT: 16.4 ± 3.6 vesicles/cell; M18OE: 13.3 ± 3.0 vesicles/cell, $p = 0.64$, Fig 4D-F) and the DCV release rate was comparable between M18OE and WT neurons (WT: 1.0 ± 0.7 vesicles/s; M18OE 0.7 ± 0.2 vesicles/s, Fig 4D). Finally, DCV release during a 60 s recording period after stimulation was not altered by the overexpression of Munc18-1 compared to WT neurons (WT: 3.3 ± 0.8 events/cell; M18OE: 2.3 ± 0.6 events/cell, Fig 4G-H). Hence, in contrast to synaptic vesicle release and DCV release from chromaffin cells, Munc18-1 overexpression in hippocampal neurons does not result in increased DCV release.

DISCUSSION

Here we compared DCV release characteristics between immature developing (DIV4) neurons and more mature (DIV14) neurons. We show that immature neurons have less DCVs than mature neurons, and that DCV release in immature neurons requires more prolonged stimulation. Also, immature neurons release relatively more DCVs after the stimulation period when calcium levels have dropped to pre-stimulus levels compared to mature neurons. Thus, the release probability of DCVs in developing neurons is lower than in mature neurons and release is less well coupled to electrical stimulation. Interestingly, in contrast to DCV release in adrenal chromaffin cells, DCV release in immature neurons does not require the presence of Munc18-1. Together this suggests that DCV release in developing neurons may be executed by different fusion machinery than release in mature neurons. Increasing the levels of Munc18-1 in mature DIV14 neurons did not increase the number of release events. Hence, in mature neurons the endogenous levels of Munc18-1 appear to be sufficient to drive efficient DCV release.

This situation differs from chromaffin cells, where deletion of Munc18-1 as well as overexpression of Munc18-1 strongly influences the number of DCV release events (Voets et al., 2001). The fact that Munc18-1 seems dispensable for DCV release in immature neurons may be explained by the different requirements for DCVs release in mature and immature neurons. At DIV3, neurons are not fully polarized into axons and dendrites and synapse formation has not yet started. Secretory vesicle release at this stage is used to deliver guidance cues, growth factors, add membrane lipids and at a later stage, Piccolo-Bassoon transport vesicles (PTVs) transport pre-assembled active zones to help create new synapses (Shapira et al., 2003). In mature neurons, after synapse formation, DCV cargo plays key roles in modulating neurotransmission. For instance, local dendritic BDNF release influences presynaptic SV release (Kuczewski et al., 2009) and axonal oxytocin release in the central amygdala controls fear responses (Knobloch et al., 2012). DCV release in mature neurons is strongly linked to sudden increases in network activity and to control synaptic strength, therefore the timing of DCV release may be more critical than during the delivery of guidance cues to the navigating axon. To meet these specific requirements, it is plausible that DCV release might be regulated by different release mechanisms during the stages of development. This difference in DCV release characteristics may be reflected in the use of other Munc18 isoforms. Munc18-1 may

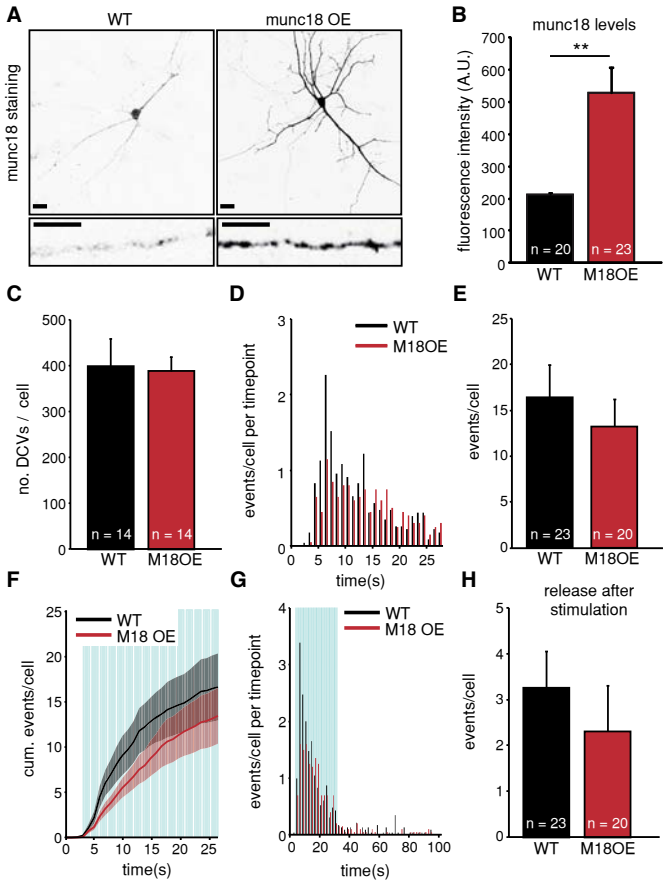
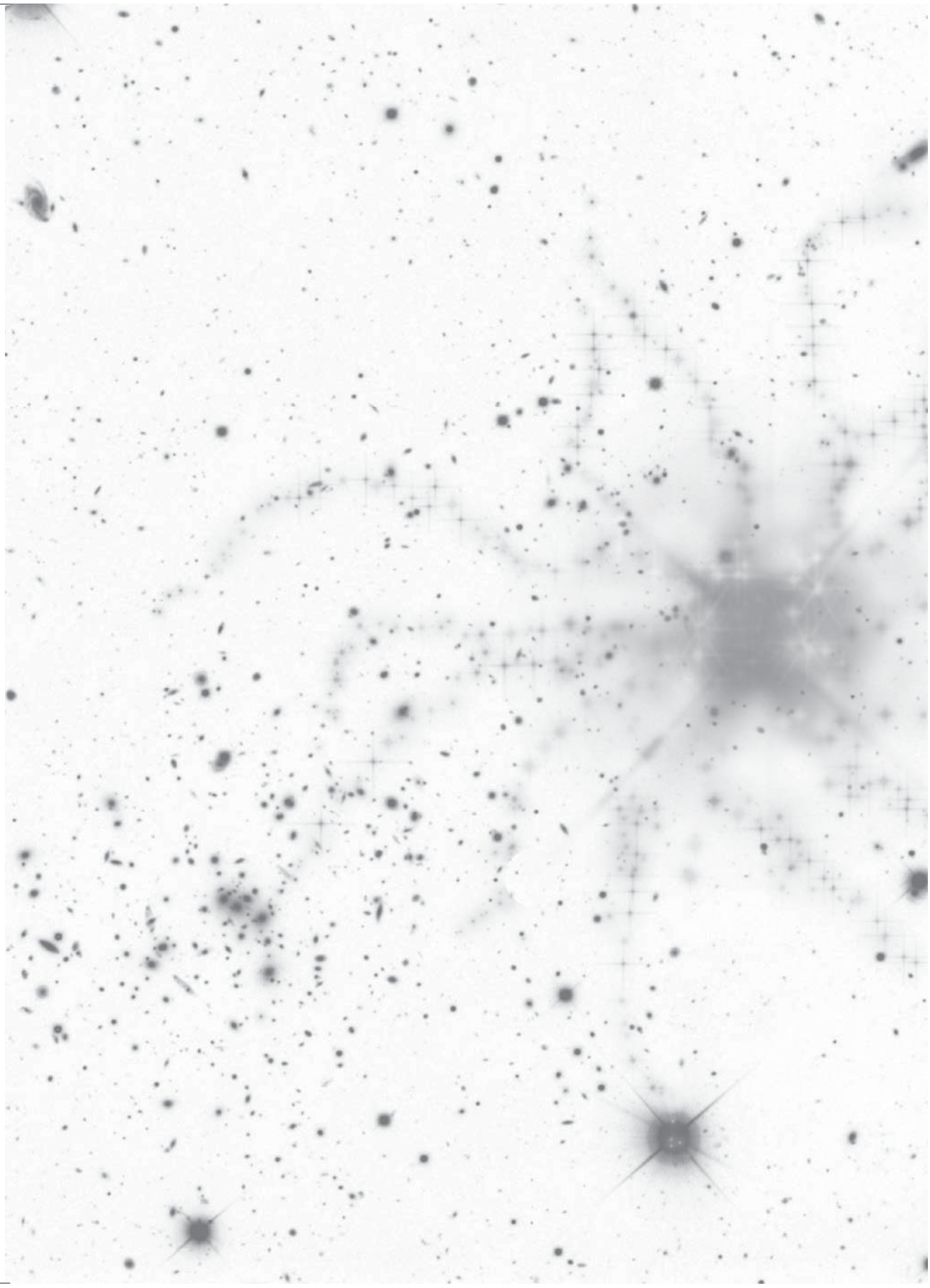


Figure 4 Overexpression of Munc18-1 does not increase DCV release in mature neurons. (A) Inverted confocal images showing Munc18-1 expression upon semi-quantitative immunofluorescence detection with an antibody against Munc18-1 in a WT and Munc18-1 overexpressing (M18OE) neuron. Zoom shows Munc18-1 expression levels in a neurite. Calibration bars are 20 µm for the whole figure and 5 µm for zooms. (B) Levels of Munc18-1 fluorescence in the neuronal cell body analyzed from confocal images (WT: n = 20 cells; M18OE: n = 23 cells, ** $p < 0.001$). (C) Total number of vesicles per cell using NH_4 application to visualize all vesicles (WT: n = 14 cells, 5579 vesicles; M18OE: n = 14 cells, 5441 vesicles, N = 3 experiments). (D) Histogram showing all release events for wildtype (WT) and WT neurons overexpressing Munc18-1 (M18OE) during the stimulation period. (E) Average number of DCV release events during stimulation for WT and M18OE neurons at DIV14 (WT: n = 23 cells, 377 events; M18OE: n = 20 cells, 265 events, N = 3 experiments). (F) Average cumulative frequency plot of DCV release events in WT and M18OE neurons at DIV14 (blue bars represent the stimulation of 16 bursts of 50 AP at 50 Hz, error bars represent SEM). (G) Histogram showing all release events for WT and M18OE neurons during the total acquisition time (blue bars represent the stimulation of 16 bursts of 50 APs at 50 Hz). (H) Number of DCV release events after the stimulation period for WT and M18OE neurons at DIV14 (WT: n = 23 cells, 75 events; M18OE: n = 20 cells, 46 events, N = 3 experiments).

control precisely timed vesicle release as for DCVs in adrenal chromaffin cells and synaptic and secretory vesicles in mature neurons. While other isoforms may be preferentially used when timing of release is less critical. Munc18-1 is the major Munc18 isoform for DCV release in chromaffin cells, but Munc18-2 can support DCV release in Munc18-1 deficient chromaffin cells albeit with strongly reduced efficiency (Gulyas-Kovacs et al., 2007). Substitution of Munc18-1 with Munc18-2 reduces the size of the primed vesicle pools even though Munc18-2 supports docking to the same extent as Munc18-1. This low release probability resembles the phenotype of DCV release in DIV 3 neurons and Munc18-2 may therefore be the most likely Munc18 isoform to support neuronal DCV release in immature neurons. The third Munc18 isoform, Munc18-3, is ubiquitously expressed with very low levels in brain and preferentially interacts with the Botulinum-insensitive syntaxin 4 (Hata and Sudhof, 1995). We have shown in Chapter 5 that neuronal DCV release is completely blocked by Botulinum application, which makes it unlikely that munc18-3 is involved in the regulation of neuronal DCV release.

Thus, Munc18-1 does not control neuronal DCV release during development and does not appear to be rate-limiting in mature neurons. Whether Munc18-1 is involved in DCV release in mature neurons is under current investigation.



CHAPTER 5

DIFFERENT SNARE COMPLEXES DRIVE NEURONAL DCV RELEASE

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ABSTRACT

SNARE protein complexes drive membrane fusion between opposing membranes. SNARE complexes consisting of syntaxin-1, SNAP25 and VAMP2 regulate synaptic vesicle (SV) fusion, but the SNARE proteins that mediate fusion of neuronal dense core vesicles (DCVs) are unknown. We show, at single vesicle resolution using neurotoxins and genetic manipulation, that DCV release from neurons relies on canonical syntaxin isoforms (syntaxin-1, -2 or -3), but functions in the absence of VAMP2, albeit slightly less efficient. DCV release is generally unaffected in mature *snap25* null mutant neurons and release in immature (DIV4) *snap25* null mutant neurons is even increased and requires less intense stimulation. Moreover, DCV release at this developmental stage is completely VAMP2 independent. This shows that the DCV release machinery in neurons differs from the machinery that drives synaptic vesicle fusion. Strikingly, during their development, neurons use different SNARE-protein combinations to drive DCV fusion.

INTRODUCTION

SNARE proteins are highly conserved proteins that are essential for the fusion of opposing membranes (Jahn & Scheller, 2006). All membrane fusion reactions, from intra-cellular fusion to exocytosis of secretory vesicles, require the formation of a tri-partite SNARE complex comprised of syntaxin, VAMP and SNAP25 isoforms. Synaptic vesicle fusion requires the neuronal SNARE complex, which consists of VAMP2, syntaxin-1 and SNAP25 (Jahn et al, 2003; Sudhof, 2004). Dense core vesicle (DCV) fusion also requires SNARE proteins. However, the composition of the SNARE complex that drives DCV fusion in neurons is not known.

DCVs transport and release a variety of cargo, molecules that are important during brain development and also function as modulatory factors of synaptic transmission in the adult brain. DCVs originate from the trans-Golgi network and are transported via microtubule-based motors to their release sites in axons and dendrites. In neuro-endocrine cells like pancreatic beta cells and adrenal chromaffin cells and at the neuromuscular junction in invertebrates like *C. elegans*, syntaxin-1 is required for DCV release (de Wit et al, 2006; Hammarlund et al, 2008; Liu et al, 2010; Ohara-Imaizumi et al, 2007). Hence, despite the presence of multiple syntaxin isoforms (-1, -2 and -4) at the plasma-membrane, the SNARE complex that drives DCV release in these systems uses the same syntaxin isoform as the SNARE complex involved in synaptic vesicle release. Botulinum toxin C (BoNT/C) cleaves syntaxin-1, -2 and 3 (Blasi et al, 1993; Schiavo et al, 1995) and thus abolishes SV and DCV release.

The vesicular SNARE protein VAMP has several isoforms (VAMP-1, -2 and 3) including a tetanus toxin insensitive variant (VAMP7 or TI-VAMP) (Galli et al, 1998). Tetanus toxin (TeNT) cleaves all isoforms of VAMP, except VAMP7, and thereby prevents the formation of fusogenic SNARE complexes. Cleaving VAMP with tetanus toxin in chromaffin cells abolishes DCV release (Hohne-Zell et al, 1994). Axonal release of the DCV cargo NT-3 is also abolished in cells treated with tetanus toxin, which suggests that VAMP is involved in DCV release in neurons (Wang et al, 2002). VAMP7 functions in axonal outgrowth in developing neurons, although *vamp7* null mutant mice show no severe developmental defects, suggesting that compensatory mechanisms ensure normal development of the brain (Danglot et al, 2012). These mice do however show abnormal anxiety behaviour, suggesting a role of VAMP7 in higher brain functions.

The SNAP protein family consists of different isoforms of which SNAP25 is important in SV fusion and SNAP23 is mostly involved in constitutive vesicle fusion (Chieregatti et al, 2004). Chromaffin cells that lack SNAP25 have a severe defect in DCV release that can partially be restored by expressing SNAP23. Because overexpression of SNAP23 in wildtype cells reduces vesicle release, SNAP23 is thought to inhibit SNAP25 dependent release in chromaffin cells by competing for a position in the SNARE complex (Sorensen et al, 2003). SNAP25 KO neurons consist of two different populations, the majority of null mutant neurons (± 98%) dies after four days in culture (DIV 4), while ± 2% survives but shows impaired arborization, reduced spontaneous release, and complete arrest of evoked release of SVs. SNAP25 KO neurons can be rescued by SNAP23, which restores spontaneous and evoked release although evoked release is highly asynchronous (Delgado-Martinez et al, 2007). Two other SNAP isoforms, SNAP29 and SNAP47 have been identified but are not yet characterized (Holt et al, 2006; Su et al, 2001). SNAP29 binds different syntaxin isoforms, of which only syntaxin-4 resides at the plasma membrane. SNAP47 binds to plasma membrane SNAREs *in vitro* but is predominantly present on intracellular membranes and therefore not a likely candidate to participate in DCV release (Bennett et al, 1993; Hohenstein & Roche, 2001; Holt et al, 2006). Since SNAP23 binds to all plasma membrane

syntaxin isoforms and because of its importance in DCV release from chromaffin cells it is a likely candidate to function in neuronal DCV release.

DCV release in chromaffin cells uses similar release machinery proteins as synaptic vesicles. Whether DCV release in neurons also relies on a similar SNARE complex is unknown. Release of DCVs in neuro-endocrine cells and neurons differ in some aspects. For instance, DCVs in neuro-endocrine cells are positioned at the plasma membrane, while DCVs in neurons are mobile and generally not in close proximity to the plasma membrane. Hence, it is conceivable that neuronal DCV release may use different release machinery composition. To test this, we used a live cell imaging approach to investigate which SNARE proteins regulate DCV release in neurons. We found that cleaving syntaxin-1, -2 and -3 with BoNT/C completely abolishes release, while cleaving VAMP-1, -2 and -3 with TeNT slightly reduces release in mature neurons with the remaining release events occurring at a slower pace. Interestingly, TeNT had no effect on DCV release in young developing neurons. The surviving population of SNAP25 KO neurons showed normal DCV release, but release required slightly prolonged stimulation. In contrast, DCV release in developing SNAP25 KO neurons was increased and required less stimulation. Thus, DCV release in neurons uses different SNARE complex members than the canonical SV SNARE complex and the DCV release complex changes composition during development.

RESULTS

DCV release is dependent on SNARE proteins

To test the involvement of the SNARE proteins VAMP and syntaxin in neuronal DCV release we used a live cell imaging approach with pH-sensitive labelled DCV cargo (DCV-pHluorin) as a tool to monitor DCV release in hippocampal neurons. To investigate the role of syntaxin in DCV release, we infected neurons at DIV14 with a semliki forest virus (SFV) expressing the light chain of Botulinum toxin C (BoNT/C). This infection protocol completely abolishes evoked synaptic vesicle release in hippocampal cultures 6 h post-infection (de Wit et al, 2006). After 6 h of SFV infection, neurons that expressed BoNT/C and DCV-pHluorin were imaged to examine DCV release. BoNT/C expression virtually abolished DCV release from these neurons (WT: 12.4 ± 3.9 vesicles/cell, n = 14 cells; BoNT/C: 0.8 ± 0.8, vesicles/cell, n = 4 cells, $^{**}p < 0.01$, Fig 1A). The few events in BoNT/C expressing cells occurred during or right after stimulation and showed a slightly increased onset time compared to wildtype neurons (WT: 12.5 ± 0.6 s, n = 258 events; BoNT/C: 18.5 ± 6.4 s, n = 3 events, Fig 1B). Hence, acute BoNT/C expression shows that neuronal DCV release is highly sensitive to BoNT/C-directed cleavage of syntaxin isoforms and suggests that the BoNT/C-substrates syntaxin-1, -2 and/or 3 are required for neuronal DCV release.

To investigate which VAMP isoform is involved in DCV release we transfected neurons at DIV10 with the light chain of tetanus toxin (TeNT) coupled to a reporter (RFP). Four days post-transfection, reporter positive cells showed a similar number of DCV release events as wildtype cells (WT: 10.3 ± 2.7 vesicles/cell, n = 10 cells; TeNT: 6.1 ± 1.2 vesicles/cell, n = 12 cells, N = 3 experiments, p = 0.18, Fig 1C). We can however not state that this time window was adequate to cleave all VAMP molecules in the whole cell. Interestingly, linear fits of the cumulative release events of TeNT transfected neurons and wildtype neurons revealed that the DCV events in TeNT transfected cells had a slower release rate (WT: 0.7 ± 0.3 vesicles/s; TeNT: 0.3 ± 0.3 vesicles/s, Fig 1D). During the first four burst of stimulation, DCV release was slightly delayed

in TeNT transfected neurons compared to wildtype neurons (WT: 5.5 ± 2.1 events/cell; TeNT: 1.1 ± 0.3 events/cell, p = 0.06, Fig 1E). In wild type cells, DCV release started at the second burst of stimulation, while release in TeNT transfected neurons required three bursts of stimulation (Fig 1F). Together, these data show that cleaving VAMP-1, -2 and -3 influences the rate of DCV release in DIV14 hippocampal neurons. This may suggest that the release observed after cleavage of VAMP-1, -2 and 3 is regulated by a different VAMP isoform with different kinetic properties.

In developing neurons, vesicle fusion is required for proper neurite outgrowth. The TeNT insensitive VAMP isoform TI-VAMP (VAMP7) is implicated in the underlying exocytic pathway although VAMP7 null mutant mice do not display significant wiring defects (Danglot et al, 2012). We investigated which VAMP isoform supports DCV release during neuronal development by analysing DCV release in DIV4 neurons. Compared to DCV release in DIV14 neurons, DIV4 neurons released less vesicles and release was not restricted to the stimulation period but continued at roughly similar rates after the stimulation ended (see wild type traces in Fig 2). This sustained

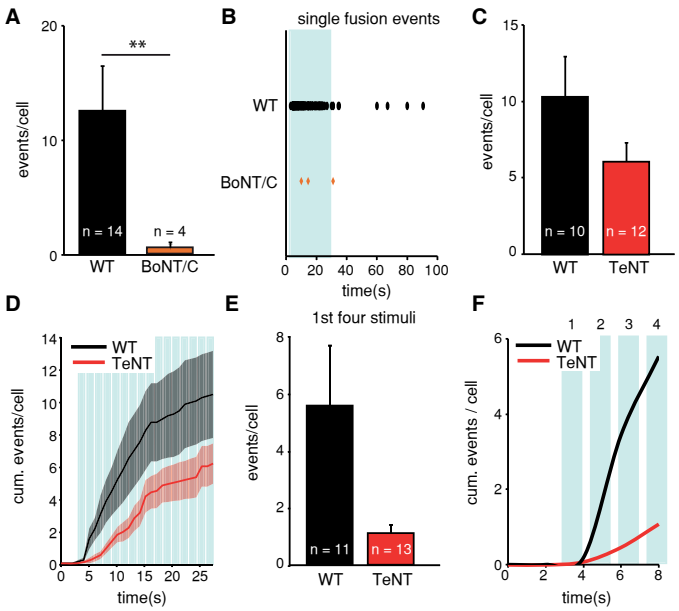


Figure 1 SNARE proteins syntaxin and to a lesser extent VAMP control DCV release in mature neurons. (A) The number of DCV release events during electrical stimulation (16 bursts of 50 AP at 50 Hz) for wildtype neurons and wildtype neurons infected with SFV BoNT/C (WT: n = 14 cells, 258 events; BoNT/C: n = 4 cells, 3 events, N = 1 experiment, **p < 0.01). (B) The individual DCV release events of wildtype and BoNT/C infected neurons plotted in time (blue bar represent stimulation of 16 burst of 50 AP at 50 Hz). (C) The number of DCV release events during electrical stimulation for wildtype neurons and wildtype neurons transfected with tetanus toxin (WT: n = 10 cells, 103 events; TeNT: n = 12 cells, 73 events, N = 3 experiments, p = 0.18). (D) Cumulative number of DCV release events for wildtype and wildtype neurons transfected with tetanus toxin during electrical stimulation. Blue bars represent the stimulation of 16 bursts of 50 AP at 50 Hz. (E) The average number of DCV release events during the first four bursts of stimulation for wildtype and tetanus toxin transfected cells (WT: n = 11 cells, 61 events; TeNT: n = 13 cells, 14 events, N = 3 experiments, p = 0.06). (F) Cumulative number of DCV release events for wildtype and tetanus toxin transfected cells during the first four bursts of stimulation (blue bars represent bursts of 50 AP at 50 Hz).

release after stimulation occurred despite the fact that calcium levels were only elevated during the stimulation period (Fig 2C). Cleaving VAMP-1, -2 and 3 did neither affect the number of DCV release events nor the timing of DCV release in TeNT expressing cells at DIV4 compared to wildtype neurons (WT: 1.7 ± 0.5 events/cell, n = 7 cells; TeNT: 2.2 ± 0.3 events/cell, n = 16 cells, p = 0.39, Fig 2A-B). Hence, DCV release in developing neurons appears to be independent of VAMP-1, -2 and 3 and therefore may be controlled by SNARE complexes containing TI-VAMP.

SNAP25 is not required for DCV release in mature neurons

We identified syntaxin isoforms -1,-2 or -3 as essential components of the SNARE complex that drives neuronal DCV release. In the SV SNARE complex, syntaxin-1 acts together with SNAP25 in calcium dependent SV release. We used SNAP25 KO mice to test whether DCVs and SVs use the same binary syntaxin-1/SNAP25 complex for release. We imaged SNAP25 KO neurons at DIV14. These neurons represent a small sub-population of SNAP25 KO neurons (2% of the total SNAP25 KO neuronal population) that are impaired in SV release and survive beyond DIV3 (Delgado-Martinez et al, 2007). In contrast to SV release, these cells showed a similar number of DCV release events compared to wildtype cells (WT: 5.2 ± 1.1 events/cell, n = 20 cells; SN25 KO: 7.7 ± 2.7 events/cell, n = 12 cells, N = 3 experiments, p = 0.41, Fig 3A). Release in SNAP25 KO neurons started at a slower release rate than WT neurons, but increased during the stimulation (< 15 s: WT 0.22 ± 0.1 vesicles/s; SN25 KO 0.12 ± 0.1 vesicles/s; > 15 s: WT 0.18 ± 0.04 vesicles/s; SN25 KO 0.48 ± 0.12 vesicles/s, Fig 3B). During the first eight bursts of 50 APs, DCV release seemed slightly lower and delayed in SNAP25 KO but is not significantly different from release in wildtype neurons (WT: 3.0 ± 0.7; SN25 KO: 1.8 ± 0.5, p = 0.41, Fig 3C-D). Hence, in contrast to SV release, DIV14 neurons do not require SNAP25 for DCV release.

SNAP25 regulates release rates at synaptic and extra-synaptic sites

SNAP25 is expressed throughout the axon but is enriched in synapses (Suh et al, 2010). Therefore, SNAP25 could affect DCV release at synapses more than at extra-synaptic sites. In

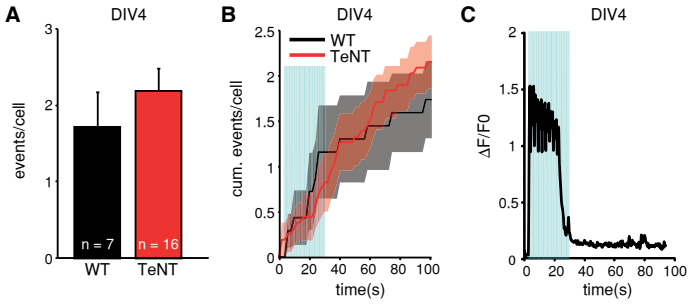


Figure 2 DCV release in developing neurons is independent of VAMP-1, -2 or -3. (A) The number of DCV release events for DIV4 wildtype neurons and wildtype neurons transfected with TeNT during the total acquisition time (WT: n = 7 cells, 12 events; TeNT: n = 16 cells, 35 events, N = 2 experiments). (B) Cumulative number of DCV release events for wildtype and wildtype neurons transfected with tetanus toxin. Blue bars represent the stimulation of 16 bursts of 50 AP at 50 Hz. DCV release in DIV4 neurons is triggered by calcium influx but continues after the end of the stimulation paradigm. (C) Calcium influx in response to electrical stimulation measured in Fluo4 loaded neurons at DIV4.

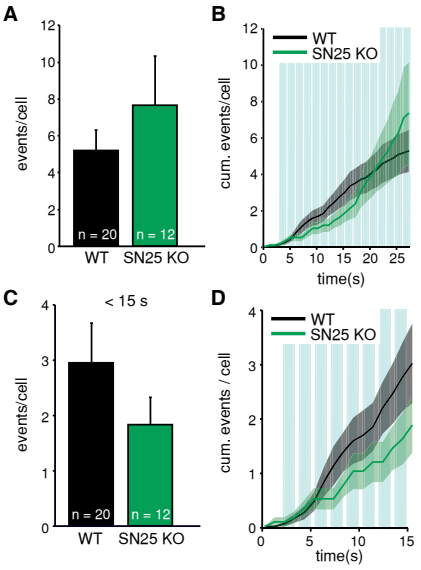


Figure 3 Neuronal DCV release from mature neurons does not require SNAP25. (A) The number of DCV release events during electrical stimulation for wildtype and SNAP25 KO neurons (WT: 104 events, n = 20 cells; SN25 KO: 92 events, n = 12 cells, N = 3 experiments). (B) Cumulative number of DCV release events for wildtype and SNAP25 KO neurons during stimulation. Blue bars represent the stimulation of 16 bursts of 50 AP at 50 Hz. (C) The number of DCV release events during the first eight bursts of stimulation for wildtype and SNAP25 KO neurons (WT: 59 events, n = 20 cells; SN25 KO: 22 events, n = 12 cells, N = 3 experiments). (D) Cumulative number of DCV release events during the first eight bursts of stimulation for wildtype and SNAP25 KO neurons. Blue bars represent the stimulation of 16 bursts of 50 AP at 50 Hz.

wildtype cells, synaptic DCV release requires less intense stimulation than extra-synaptic DCV release and the slower release rates during the first 15 seconds of stimulation in SNAP25 KO neurons (Fig 4B-C) could thus result from the loss of synaptic release events. Therefore, we analyzed which DCV release events occurred at synapses by measuring the co-localization with the synaptic marker synapsin. In wildtype neurons, the majority of DCV release events occurred at synaptic sites (WT: 68% ± 7.5, n = 23 cells, N = 3 experiments, Fig 4A). This was unchanged in SNAP25 KO neurons (SN25 KO: 70% ± 5.6, n = 14 cells, N = 3 experiments, Fig 4A). However, synaptic release in SNAP25 KO neurons required prolonged stimulation compared to wild type neurons (Fig 4B and D) while extra-synaptic release was less affected (Fig 4C). These data show that SNAP25 deletion does not affect the location of DCV release. It does however make DCV release less efficient requiring prolonged stimulation specifically at synaptic sites.

SNAP25 is not required for DCV release in developmental neurons

We also investigated the effect of SNAP25 deletion on DCV release in developing neurons at DIV4. Similar to wild type neurons, SNAP25 KO neurons at DIV4 also continue to release DCVs after the stimulation at similar rates as during stimulation (Figure 2 and 5B). At this stage, SNAP25 KO neurons are indistinguishable from wild type cells with cell death starting from DIV5-7 onwards. SNAP25 KO neurons at DIV3 release twice the amount of DCVs upon stimulation compared to wildtype (WT: 5.3 ± 1.5 events/cell, n = 17 cells; SN25 KO: 10 ± 2.3 events/cell, n = 16 cells, $^{*}p < 0.05$, Fig 5A). In developing SNAP25 KO neurons, DCV release is faster than in developing WT neurons and requires less intense stimulation (WT: 0.06 ± 0.1 vesicles/s, SN25 KO: 0.1 ± 0.5 vesicles/s, Fig 5B). Although SNAP25 KO neurons release more DCVs per stimulation burst than wildtype neurons, both start releasing DCVs at the same time (Fig 5C). These data show that in developing neurons, DCV release is not dependent on SNAP25, and suggest that other SNAP isoforms are involved in DCV release at this developmental stage.

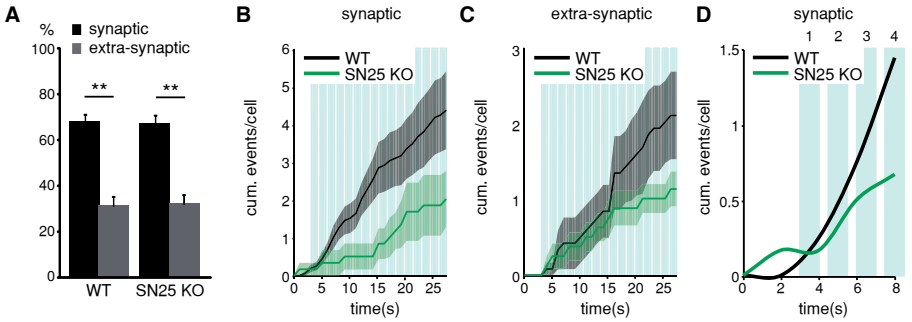


Figure 4 SNAP25 does not control synaptic preference of DCV release. (A) Percentage of DCV release events co-localizing with synapsin puncta in WT cells and SNAP25 KO cells, **p < 0.01. (B) Cumulative number of synaptic DCV release events for wildtype and SNAP25 KO neurons. Blue bars represent the stimulation of 16 bursts of 50 AP at 50 Hz. (C) Cumulative number of extra-synaptic DCV release events for wildtype and SNAP25 KO neurons. Blue bars represent the stimulation of 16 bursts of 50 AP at 50 Hz. (D) Cumulative number of synaptic DCV release events per cell for wildtype and SNAP25 KO neurons during the first four bursts of stimulation (blue bars represent bursts of 50 AP at 50 Hz).

SNAP23 expression fluctuates during neuronal development

The ubiquitously expressed SNAP isoform SNAP23 shows high sequence homology with SNAP25 (Ravichandran et al, 1996). SNAP23 can form a SNARE complex with syntaxin-1 and might therefore be involved in DCV release in SNAP25 KO neurons. To test this, we first investigated SNAP23 localization in wildtype neurons. This revealed that SNAP25 and SNAP23 do not co-localize: SNAP25 was expressed mainly in axons and synapses, while SNAP23 co-localized with MAP2 expression and hence was mainly dendritically expressed (Fig 6A). This non-overlapping expression in wildtype neurons makes it less likely that SNAP23 can replace SNAP25 to drive DCV release in mature neurons although we did not test SNAP23 expression in the absence of SNAP25.

Both SNAP23 and SNAP25 expression increased over time in cultured wild type neurons (Fig 6B). As SNAP23 over-expression can rescue the lethality of SNAP25 KO neurons (Delgado-Martinez et al, 2007), we wondered why the majority of SNAP25 KO neurons died between DIV4-6. A possible explanation may be that SNAP23 levels fluctuate during development and reach a critically low level around DIV4 that cannot support cell viability in the absence of SNAP25. The remaining 2% of SNAP25 KO neurons that do survive could represent a sub-population expressing enough SNAP23 during this critical period. To test this, we stained SNAP25 KO neurons at different time points for SNAP23. We found that SNAP23 expression levels indeed drop around DIV4 and show increased expression in surviving SNAP25 KO neurons at DIV7 and DIV15 (Fig 6C). This supports the hypothesis that neurons need high enough levels of SNAP23 for survival.

DISCUSSION

Membrane fusion in eukaryotic cells is regulated by SNARE complexes. The segregation of different SNARE isoforms on specific cellular compartments ensures that fusion occurs at the correct place. Here we find that neuronal DCV release is regulated by syntaxin-1, -2 or -3, but that DCV release is possible in the absence of VAMP-1, -2 and -3 and SNAP25. This suggests

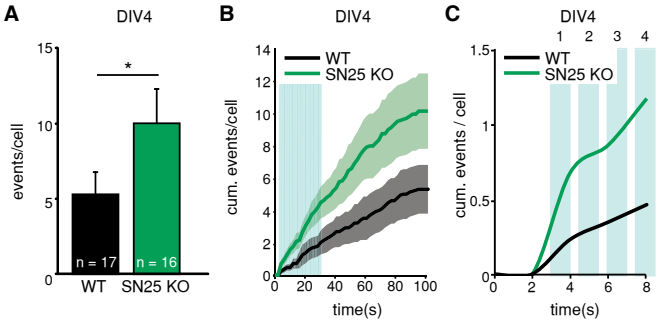


Figure 5 SNAP25 deletion in developing neurons results in increased DCV release. (A) The number of DCV release events in developing neurons (DIV4) for wildtype and SNAP25 KO neurons (WT: 90 events, n = 17 cells; SN25 KO: 160 events, n = 16 cells, N = 3 experiments, *p < 0.05). (B) Cumulative number of DCV release events for wildtype and SNAP25 KO neurons. Blue bar represents the stimulation of 16 bursts of 50 AP at 50 Hz. (C) Cumulative number of DCV release events for wildtype and SNAP25 KO neurons during the first four bursts of stimulation (blue bars represent bursts of 50 AP at 50 Hz).

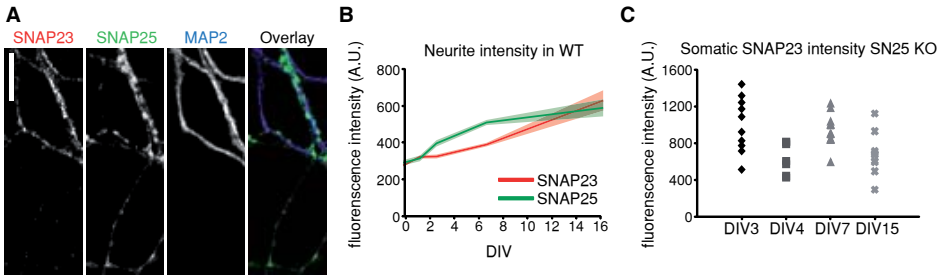


Figure 6 Fluctuations in SNAP23 expression levels may explain cell death in SNAP25 null mutant neurons. (A) Confocal image of a WT neuron stained for SNAP23 (red), SNAP25 (green) and the dendritic marker MAP2. Scale bar represents 10 µm. (B) Fluorescence intensity of SNAP23 or SNAP25 antibody in neurites of wildtype cells from DIV2 to DIV14 (n = 10 cells per DIV). (C) Fluorescence intensity of SNAP23 or SNAP25 in the soma of SNAP25 KO neurons plotted for each cell individually (n = 10 cells per DIV). Most (98%) SNAP25 KO neurons die between DIV3 and DIV4 and only 2% survives beyond DIV4; DIV7 and DIV15 represent these surviving neurons.

that other complexes than the canonical SV SNARE complex regulate DCV release. In addition, during their development, neurons use different SNARE-protein combinations to fuse DCVs.

Neurons express many isoforms of syntaxin, although only four (syntaxin-1, -2, -3 and -4) are expressed at the plasma membrane. Botulinum toxin C cleaves three of these isoforms (syntaxin-1, -2 and -3). Although syntaxin-1 is the most likely syntaxin to drive neuronal DCV release, our experiments do not rule out a role for syntaxin-2 or -3. Syntaxin-2 functions together with SNAP23 and cellubrevin in release of alpha-granules from blood platelets (Feng et al, 2002). Syntaxin-3 functions in epithelial cell polarity and membrane expansion (Darios & Davletov, 2006) and knockdown of syntaxin-3 in mouse pancreatic cells inhibits insulin release (Zhu et al, 2012). The expression of splice variant syntaxin 2D is restricted to brain (Quinones

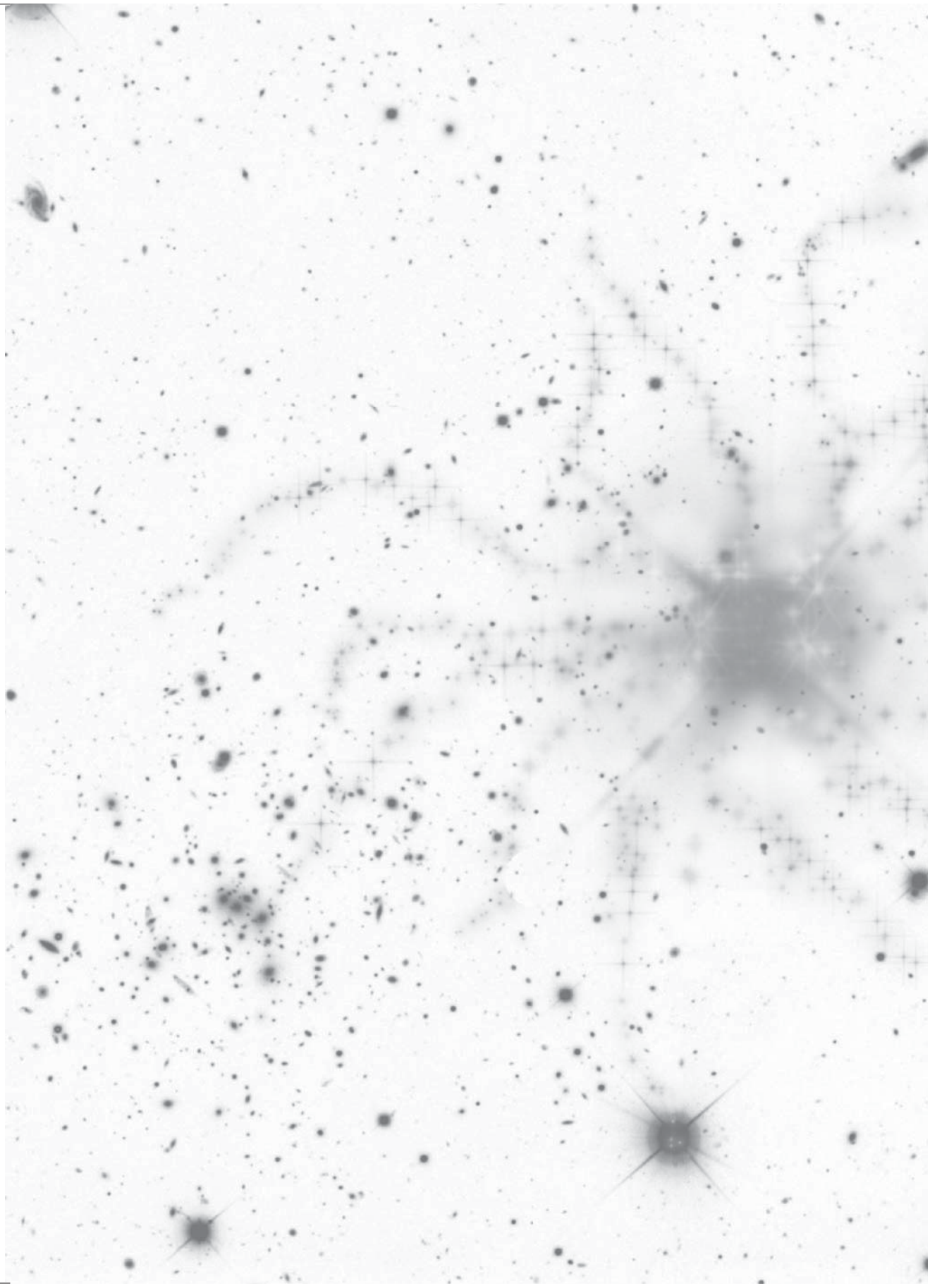
et al, 1999) and syntaxin-3 is expressed in brain as well, with increased expression in the retina (Morgans et al, 1996). Hence, all three syntaxin isoforms (-1, -2 and -3) are candidates for participating in neuronal DCV release.

Interestingly, syntaxin-4, which is not affected by BoNT/C, is localized at the post-synaptic membrane and defines sites for activity-dependent exocytosis of glutamate receptors (Kennedy et al, 2010). We found that cleaving syntaxin-1, -2 and -3 completely abolishes DCV release, thus showing that syntaxin-4 is not essential for DCV release, also not at post-synaptic sites. Hence, dendritic spines use two different SNARE complexes to fuse secretory vesicles; one complex drives membrane incorporation of glutamate receptor vesicles while the other is required for postsynaptic DCV release.

Tetanus toxin cleaves VAMP-1, -2 and -3, but does not affect tetanus toxin insensitive TI-VAMP (VAMP7). We found that TeNT expression slightly reduced the amount of DCV release in mature neurons, but did not abolish release. TeNT is a very effective toxin and application leads to a full arrest of SV fusion (de Wit et al, 2009), it is therefore unlikely that the remaining DCV release events resulted from incomplete cleavage of VAMP-1, -2 or -3. This suggests that other tetanus insensitive VAMPs regulate DCV release, but at a somewhat slower rate (Figure 1D, F). VAMP7 is tetanus toxin insensitive and implicated in the release of vesicles that support axonal outgrowth during development. VAMP7 is also expressed in hippocampal neurons at DIV14 (Coco et al, 1999) and *VAMP7* null mutant mice show increased anxiety suggesting a role for VAMP7 in higher brain functions (Danglot et al, 2012) perhaps by regulating DCV release. We found that in developing neurons, VAMP-1, -2 and -3 are not involved in DCV release and again VAMP7 is a likely candidate to regulate DCV release at this age. Another TeNT insensitive VAMP isoform is VAMP4, which is involved in the transport from early endosomes to the trans-Golgi network (TGN) (Mallard et al, 2002). Recent data however, suggested a role for VAMP4 at the synaptic terminal supporting asynchronous release of synaptic vesicles (Raingo et al, 2012). Future experiments should investigate whether the slower DCV release remaining after TeNT application in adult neurons is indeed regulated by VAMP7 or VAMP4.

SNAP25 KO neurons consist of two different populations: the majority (±98%) dies around DIV4, while ±2% survives and develops into mature neurons that are not capable of SV release. We investigated DCV release in both populations. In mature neurons lacking SNAP25, DCV release is not abolished, but DCV release is much slower compared to wildtype. Synaptic preference is not affected but especially at synapses, DCV release requires prolonged stimulation compared to wild type. Thus, a SNAP25 deficient SNARE complex in mature neurons supports DCV not as efficient as a complex with SNAP25. In contrast, in developing neurons lacking SNAP25, the number of DCV release events was significantly increased compared to wildtype. Hence, at this age SNAP25 deficient SNARE complexes function better to support DCV release than SNAP25 containing complexes. These phenotypes are remarkably different from the almost complete absence of DCV release in embryonic chromaffin cells of SNAP25 KO mice (Sorensen et al, 2003). This supports the notion that DCV release in neurons is mechanistically distinct from DCV release in neuro-endocrine cells. Especially the gain-of-function phenotype in developing SNAP25 KO neurons is remarkable. To date no SNAP25 isoform is known that is more efficient in releasing secretory vesicles. In chromaffin cells, SNAP23 can participate in DCV release and substitute for SNAP25, albeit with strongly reduced efficiency (Sorensen et al, 2003). Other SNAP isoforms expressed in brain are unlikely candidates to participate in DCV release as

SNAP29 does not bind to syntaxin-1, -2 or 3 (Hohenstein & Roche, 2001) and SNAP47 is localized on intracellular membranes and not at the plasma membrane (Holt et al, 2006). Hence, SNAP23 remains the most likely candidate to drive neuronal DCV release in the absence of SNAP25. The molecular mechanism underlying the increased release efficiency in developing SNAP25 KO neurons remains unknown. Possibly, SNAP23 engages with VAMP isoforms that are not present in chromaffin cells, to form functional and more efficient SNARE complexes to drive neuronal DCV release during development. A SNAP23/VAMP7 complex together with syntaxin-1 might be such an efficient SNARE complex that due to the developmental expression of VAMP7 specifically functions during early development. Future experiments using VAMP7 null mutant neurons and SNAP23 knock down approaches will shed light on this issue.



CHAPTER 6

DOC2B IS A HIGH-AFFINITY CALCIUM SENSOR FOR NEURONAL DCV RELEASE

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ABSTRACT

Activity-dependent release of neuropeptides from dense core vesicles (DCVs) requires a calcium sensor that triggers vesicle fusion upon calcium influx. The synaptic vesicle membrane protein, synaptotagmin-I (Syt-I), binds calcium and plays a critical role during synchronous release of synaptic vesicles. However, the calcium sensor for neuronal DCV release is unknown. Here, we investigated DCV release in different calcium sensor mutants. We show that, in contrast to synaptic vesicle (SV) release, the low affinity calcium sensor Syt-I is not involved in DCV release. In contrast, the high-affinity calcium-binding double C2 domain (Doc2) proteins that act as Ca^{2}+ sensors for spontaneous SV release also control DCV release. In *Doc2* null mutant neurons, DCV release is reduced by 50% and synaptic preference of DCV release is lost. Hence, spontaneous SV and evoked DCV release appear to rely on similar high-affinity calcium sensing mechanisms.

INTRODUCTION

Calcium dependent release of secretory vesicles is essential for cellular communication. Calcium sensing proteins trigger release by Ca^{2}+-dependent interactions with both the fusing phospholipid membranes and the membrane fusion machinery, where their calcium binding properties determine the kinetics of vesicle secretion (Burgoyne and Morgan, 1998; Chapman, 2008). Many different types of calcium sensors exist with different calcium affinities and localization (Pang and Sudhof, 2010). In this study we investigated the role of two calcium sensors, synaptotagmin-I (Syt-I) and Doc2, in neuronal dense core vesicle (DCV) release. Syt-I is a low affinity calcium sensor (mM range) and is localized at the vesicular membrane (Brose et al., 1992). Doc2 is a cytoplasmic calcium sensor with high calcium affinity (μM range) that translocates to the plasma membrane upon calcium influx (Groffen et al., 2006).

Syt-I is the main calcium sensor for synaptic vesicle (SV) release. Genetic deletion of Syt-I results in a specific loss of synchronous SV release and an increase in spontaneous release (Geppert et al., 1994; Xu et al., 2009). This increase may be the result of removing Syt-I's function as fusion clamp at low calcium levels (Chicka et al., 2008). Calcium influx converts Syt-I from a fusion clamp to a trigger for evoked release of SVs, suggesting a dual role for Syt-I (Walter et al., 2011). In the absence of Syt-I, other sensors sensitive enough to function at resting state calcium levels could drive spontaneous SV release. Doc2 is such a high affinity calcium sensor that indeed regulates spontaneous SV release (Groffen et al., 2010). The two isoforms of Doc2, Doc2a and Doc2b, are expressed in the brain with very little co-localization and with different calcium and lipid binding properties (Groffen et al., 2006). Importantly, deletion of Doc2b strongly reduces spontaneous SV release (Groffen et al., 2010; Pang et al., 2011).

Information on calcium sensor function in neuronal DCV release is scarce. Synaptotagmin IV (Syt-IV) localizes to BDNF containing DCVs. In *Syt-IV* null mutant neurons, BDNF release is slightly increased (±20%) suggesting that Syt-IV inhibits rather than promotes release of neuronal DCVs (Dean et al., 2009). Most current data on calcium sensors in DCV release comes from non-neuronal systems. Chromaffin cells use both Syt-I and synaptotagmin-7 (Syt-7) for release of DCVs and deletion of both genes completely eliminates the fast exocytotic burst (Schonn et al., 2008). Hence, in chromaffin cells, Syt-I and Syt-7 appear to be the main calcium sensors for synchronous DCV release. The effect of Doc2b deletion on DCV release in chromaffin cells is unknown, but Doc2b overexpression strongly increases DCV release from these cells and the calcium dependent translocation of Doc2b to the plasma membrane affects DCV priming (Friedrich et al., 2008). Doc2b overexpression also increases DCV release form pancreatic β-cells (Ke et al., 2007; Miyazaki et al., 2009) and adipocytes (Fukuda et al., 2009), while Doc2a overexpression enhances secretion of exogenous growth hormone in PC12 cells (Orita et al., 1996). Hence, both Syt and Doc2 proteins appear to be involved in the regulation of DCV release from (neuro-) endocrine cells.

DCV release from adrenal chromaffin cells uses similar molecular machinery as SV release and DCV release has been used extensively as a model system for SV release. Whether chromaffin DCV release is a good model for release of DCVs from central neurons is a matter of debate. While these two systems share similarities, there are also clear differences. For instance, the majority of neuronal DCVs are transported via the tubulin network with, in contrast to DCVs in chromaffin cells, only a few DCVs docked at the plasma membrane. Thus, the majority of neuronal DCVs is not closely localized to synaptic calcium channels (Chapter 2 of this thesis). Therefore, it is conceivable that neuronal DCVs use a different mechanism of

calcium sensing than SVs and DCVs in neuro-endocrine cells. To test this, we investigated the role of Syt-I and Doc2 in neuronal DCV release. We used *synaptotagmin 1* null mutant mice (Syt-I KO) and *Doc2a/b* double null mutant mice (Doc2a/b DKO) in a live cell imaging approach to study release at single vesicle resolution. We found that DCV release is not dependent on Syt-I. Doc2a/b deletion however results in a strong reduction in the number of DCV fusion events and synaptic preference of DCV release is lost. Doc2 moves to the membrane upon stimulation and could support the recruitment of DCVs to fusion sites.

RESULTS

Synaptotagmin-I deletion does not affect DCV release

To investigate the role of Syt-I in DCV release from neurons, we used Syt-I KO neurons and studied single DCV release events upon electrical field stimulation using a paradigm of 16 burst of 50 AP at 50 Hz as described in Chapter 2. Syt-I deletion did not affect the average number of DCV release events per cell compared to WT neurons (WT 14.4 ± 3.8 events/cell; Syt-I KO 10.2 ± 1.4 events/cell, Fig 1A, $p = 0.31$). The cumulative plot of the average number of DCV release events over time showed highly similar release rates between Syt-I KO neurons and WT neurons (WT 0.6 ± 0.7 vesicles/s; Syt-I KO 0.5 ± 0.4 vesicles/s, Fig 1B). We zoomed in on the first four 50 Hz bursts of the stimulation because the effect of deletion of a calcium sensor on release is likely most prominent here. Deletion of Syt-I did not affect the average number of DCV release events per cell during these first four bursts compared to WT neurons (WT 1.7 ± 0.7 events/cell; Syt-I KO 2.3 ± 0.7 events/cell, Fig 1C, $p = 0.37$). Both in WT neurons and Syt-I KO neurons, DCV release started at the second stimulation burst and showed similar release rates (WT 0.2 ± 0.2 vesicles/s; Syt-I KO 0.5 ± 0.2 vesicles/s, $p = 0.48$, Fig 1D). Hence, deletion of Syt-I does not influence the total number and rate of DCV release events in hippocampal neurons.

Synaptotagmin-I deletion does not alter synaptic preference of DCV release

Neuronal DCVs are secreted from synaptic and extra-synaptic regions at the plasma membrane. Because Syt-I is enriched in synapses, loss of this calcium sensor could potentially influence the number of synaptic DCV release events. However, Syt-I deletion did not affect the ratio of synaptic versus extra-synaptic release events (Fig 2A). Despite the fact that Syt-I is strongly enriched in synaptic terminals, Syt-I deletion did not affect synaptic release but slightly decreased extra-synaptic release (WT 195 synaptic events, 91 extra-synaptic events; Syt-I KO 201 synaptic events, 65 extra-synaptic events, Fig 2B) and reduced extra-synaptic release rates compared to WT neurons (WT 0.3 ± 0.2 vesicles/s; Syt-I KO 0.1 ± 0.08 vesicles/s, Fig 2C), while synaptic release rates were not affected (WT 0.4 ± 0.5 vesicles/s; Syt-I 0.4 ± 0.3 vesicles/s, Fig 2D). Hence, deletion of Syt-I does not reduce the number of DCV release events at synapses nor does it affect synaptic DCV release rate. However, deletion of Syt-I appears to affect release rates at extra-synaptic sites.

Doc2 affects the amount and location of DCV release

Doc2 is a high affinity calcium sensor that binds to lipid membranes upon calcium binding. Because of its high calcium affinity and cytoplasmic localization, Doc2 could function as calcium sensor for distant DCVs. To test this, we compared DCV release in Doc2 DKO mice, heterozygote littermates (Doc2a/b hetero) and wildtype mice. Deletion of both Doc2a and Doc2b resulted in a

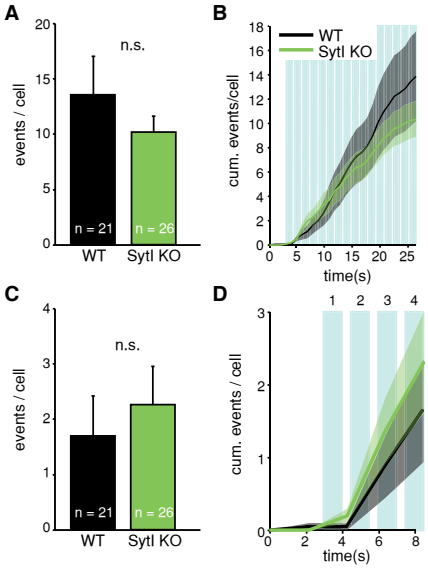


Figure 1 Synaptotagmin-I is not involved in DCV release. (A) DCV release events for WT and Synaptotagmin-I KO cells during the stimulation period (WT 21 cells, 288 release events; Sytl KO 26 cells, 285 release events, N = 3, p = 0.31). (B) The average cumulative frequency of DCV release events per cell during stimulation in WT compared to Syt-I KO neurons (the blue bars represent 16 bursts of 50 AP at 50 Hz, error bars represent SEM). (C) Average number of release events during the first four bursts for WT and Syt-I KO neurons (p = 0.37). (D) Zoom of the average cumulative frequency of DCV release during the first four bursts of stimulation (blue bars 1, 2, 3, 4 each represent 50 AP at 50 Hz) for WT and Syt-I KO neurons.

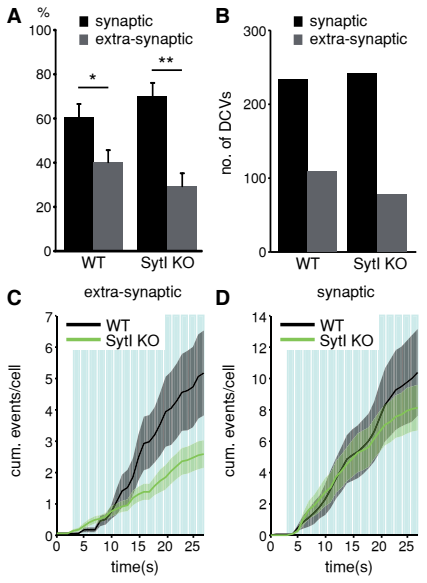


Figure 2 Synaptotagmin-I deletion does not alter synaptic preference of DCV release. (A) Percentage of DCV release events colocalizing with synapsin puncta in WT neurons and Syt-I KO neurons (*p < 0.05, **p < 0.01, ttest) (B) Absolute numbers of synaptic and extra-synaptic release events for three datasets of WT and Sytl KO neurons showing an increase of synaptic events and a loss of extra-synaptic events (WT 195 synaptic events, 91 extra-synaptic events, 21 cells; Syt-I KO 201 synaptic events, 65 extra-synaptic events, n = 26 cells, N = 3). (C) Cumulative frequency of synaptic DCV release events per cell in Syt-I KO neurons compared to WT neurons during the entire stimulation period (WT 21 cells; Syt-I KO 26 cells). (D) Cumulative frequency of extra-synaptic DCV release events per cell in Syt-I KO neurons compared to WT neurons during the entire stimulation period (error bars represent SEM).

reduced number of DCV release events compared to Doc2a/b hetero neurons (Doc2a/b hetero 10.8 ± 5.4 events/cell; Doc2 DKO 5.6 ± 1.5 events/cell, p = 0.4, Fig 3A). Most likely due to the low number of cells, this did not reach statistical significance. However, Doc2 DKO neurons showed strongly reduced DCV release compared to WT neurons (p = 0.02). The DCV release rate in Doc2 DKO neurons was lower than in control neurons (Doc2a/b hetero 0.4 ± 0.4 vesicles/s; Doc2 DKO

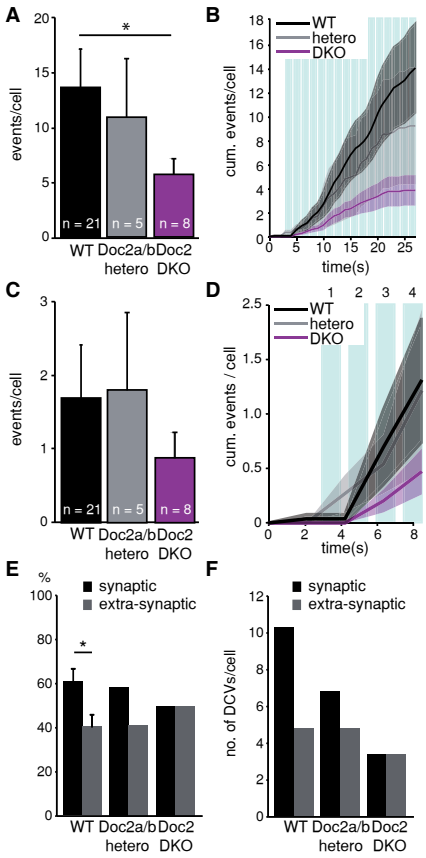


Figure 3 Doc2 is important for DCV release. (A) DCV release events for Doc2a/b hetero cells and Doc2 DKO cells during the stimulation period (Doc2 hetero 60 release events, N = 1 experiment; Doc2 DKO 31 release events, N = 2 experiments) compared to WT cells (WT 21 cells, 288 release events). (B) Average cumulative frequency plot showing the onset time of the DCV release events per cell during stimulation of WT and Doc2 hetero compared to Doc2 DKO cells, the blue bars represent 16 bursts of 50 AP at 50 Hz. (C) Average number of release events for the first four bursts for WT, Doc2a/b hetero neurons and Doc2 DKO neurons. (D) Zoom of the average cumulative frequency of DCV release during the first four bursts of stimulation (blue bars 1, 2, 3, 4 each represent 50 AP at 50 Hz) for WT, Doc2a/b hetero neurons and Doc2 DKO neurons. (E) Percentage of DCV release events colocalizing with synapsin puncta in WT, Doc2a/b hetero and Doc2 DKO neurons (*p<0.05). (F) Number of synaptic and extra-synaptic events for WT, Doc2a/b hetero or Doc2 DKO cells, corrected for the number of cells per group.

0.2 ± 0.1 vesicles/s, WT: 0.6 ± 0.7 vesicles/s, Fig 3B). Deletion of both Doc2 isoforms also reduced DCV release events during the first four bursts of stimulation but this did not reach statistical significance (Doc2a/b hetero 1.8 ± 1.1 events/cell; Doc2 DKO 0.9 ± 0.4, WT 1.7 ± 0.7 events/cell, p = 0.46, Fig 3C). DCV release required slightly more prolonged stimulation in Doc2 DKO neurons with release occurring only after the second train of stimulation, while in Doc2a/b hetero and WT neurons release started after the first 50 Hz train of stimulation (Fig 3D).

DCV release in WT and Doc2a/b heterozygote neurons showed a preference for synaptic sites. Interestingly, in Doc2 DKO neurons this preference was lost (Fig 3E). Both synaptic and extra-synaptic events were reduced in Doc2 DKO neurons (Doc2a/b hetero 6.8 synaptic events/cell; 4.8 extra-synaptic events/cell; Doc2 DKO 3.4 synaptic events/cell, 3.4 extra-synaptic events/cell, Fig 3F). Thus, although more cells have to be included in future studies, Doc2 proteins appear to regulate DCV release and affect the synaptic preference of these release events.

Doc2 translocates to the plasma membrane upon stimulation

Doc2 moves to the plasma membrane upon calcium influx in HEK cells and neurons (Groffen et al., 2006). In neurons, Doc2 translocates in response to bursts of action potentials, and higher frequencies (10-40 Hz) appear to be more effective than lower frequencies (2-5 Hz) (Groffen et

al., 2006). To investigate how Doc2 responds to our stimulation protocol of 16 bursts of 50 APs at 50 Hz, we expressed Doc2b-RFP in WT hippocampal neurons and imaged its localization before and during stimulation. Doc2b-RFP showed a homogenous distribution throughout neurites before stimulation, but rapidly moved to the plasma membrane at the onset of the stimulus train (Fig 4A-B). During the stimulation protocol, Doc2b moved from and to the membrane during each 50 AP burst (Fig 4C), but RFP fluorescence never completely returned to baseline in between bursts, suggesting that a portion of Doc2b remained at the plasma membrane during the inter-stimulus intervals.

DISCUSSION

Here we find that deletion of the main calcium sensor for SV release and DCV release in chromaffin cells, Syt-I, does not significantly affect DCV release in neurons. Deletion of both Doc2 proteins did result in lower DCV release numbers and release rates, and abolished synaptic preference of DCV release. This shows that, like spontaneous SV release, activity-dependent DCV release in mature neurons requires a high affinity calcium sensor.

Our data show that Syt-I is not a main contributor to DCV release in neurons, which is in apparent contrast with data from chromaffin cells where Syt-I deletion strongly affects the rapid release of DCVs residing in the readily releasable pool (RRP) (Voets et al., 1999). This RRP represents the primed and pre-docked DCVs that are likely in close proximity to calcium channels and that are released upon acute high calcium levels. DCVs in neurons are generally not pre-docked and do not typically reside in close proximity of calcium channels. We and others indeed show that neuronal DCV release is triggered by more prolonged and global rises in calcium levels (Chapter 2 and Hartmann et al., 2001; Matsuda et al., 2009). Interestingly, DCV release triggered by prolonged stimulation in chromaffin cells, like the sustained phase upon calcium uncaging or repetitive depolarizations, is also not affected by Syt-I deletion (Voets et al, 1999). Hence, both in neurons and chromaffin cells, Syt-I functions in synchronous release

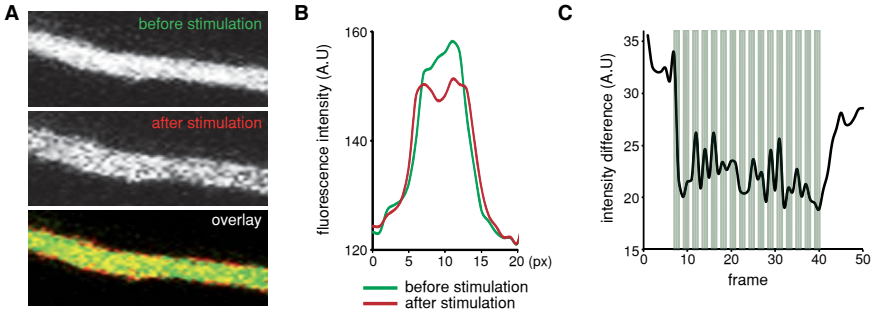


Figure 4 Doc2 moves to membrane upon stimulation. (A) Two subsequent frames of a time-lapse (2 Hz) of a neuron that expresses Doc2b-RFP stimulated with 16 burst of 50 AP at 50 Hz. Before stimulation, Doc2b is diffuse throughout the neurite. Already at the first frame after stimulation Doc2b-RFP moves to the plasma membrane. (B) Linescan of the neurite in A, showing the distribution of the Doc2b-RFP fluorescence upon stimulation. (C) Graph showing the intensity difference between the plasma membrane and the centre of the neurite for every frame. Green bars represent the stimulation of 16 bursts of 50 AP at 50 Hz.

of primed vesicles from the RRP, where sustained release during more global calcium rises likely requires high-affinity calcium sensors.

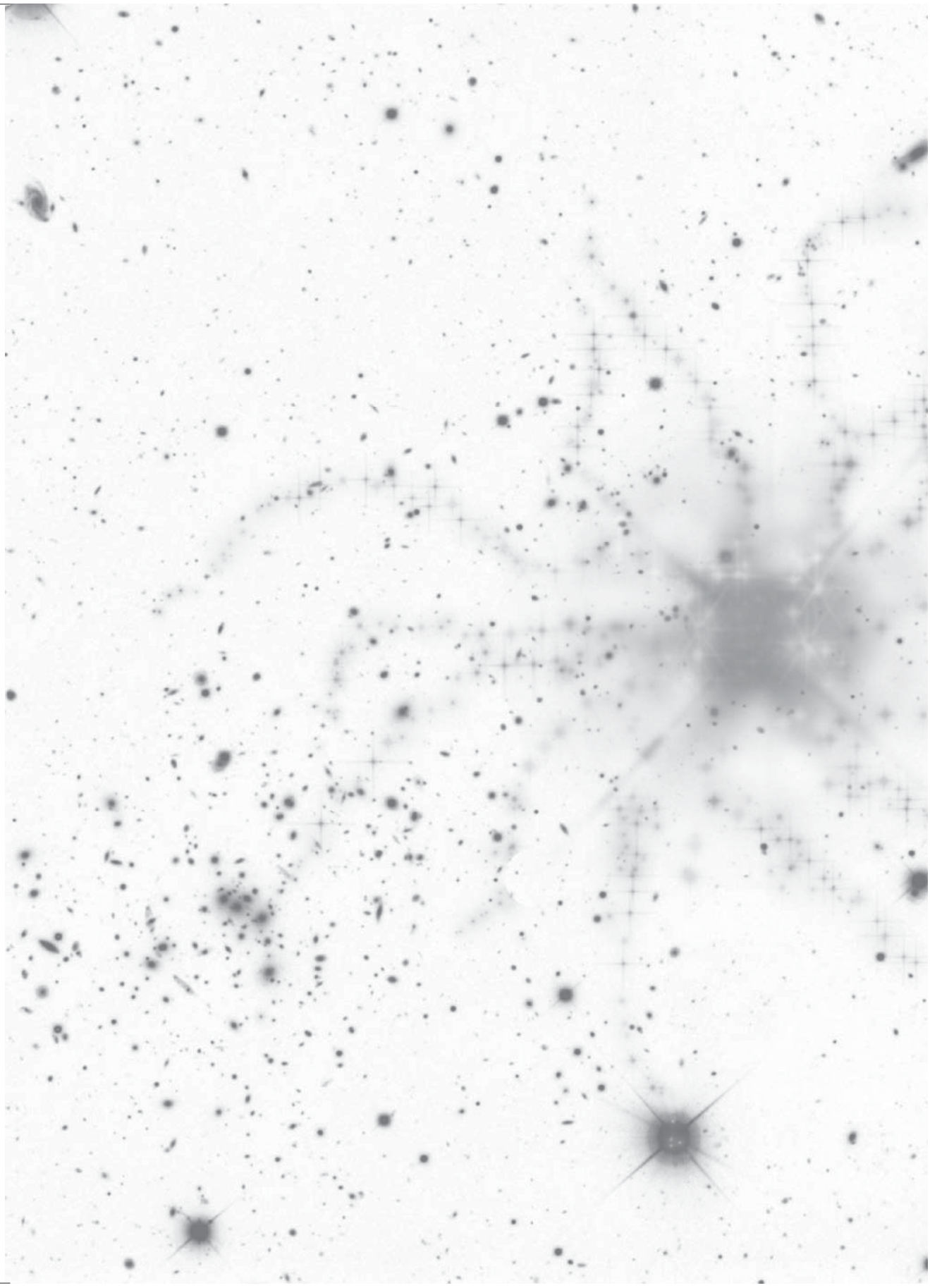
We found that the high-affinity calcium sensor Doc2 is involved in the regulation of DCV release in neurons. Its deletion affected both the number and location of DCV release. How might Doc2 function in DCV release? Upon calcium influx, Doc2 accumulates at the plasma membrane together with the priming factor munc13-1 (Duncan et al., 1999). Inhibiting the interaction between Doc2 and munc13 reduces calcium-dependent exocytosis from PC12 cells (Orita et al., 1996). Hence, Doc2 and munc13 proteins may function together to support neuronal DCV release. Deletion of Doc2 (this chapter) or munc13 (Chapter 2) results in a 50% reduction of release events and affects the location of DCV release. Munc13 is strongly enriched at synaptic terminals (Kalla et al., 2006), which suggests that munc13 may recruit Doc2 to synaptic terminals upon calcium influx thereby creating fusogenic complexes that facilitate DCV fusion.

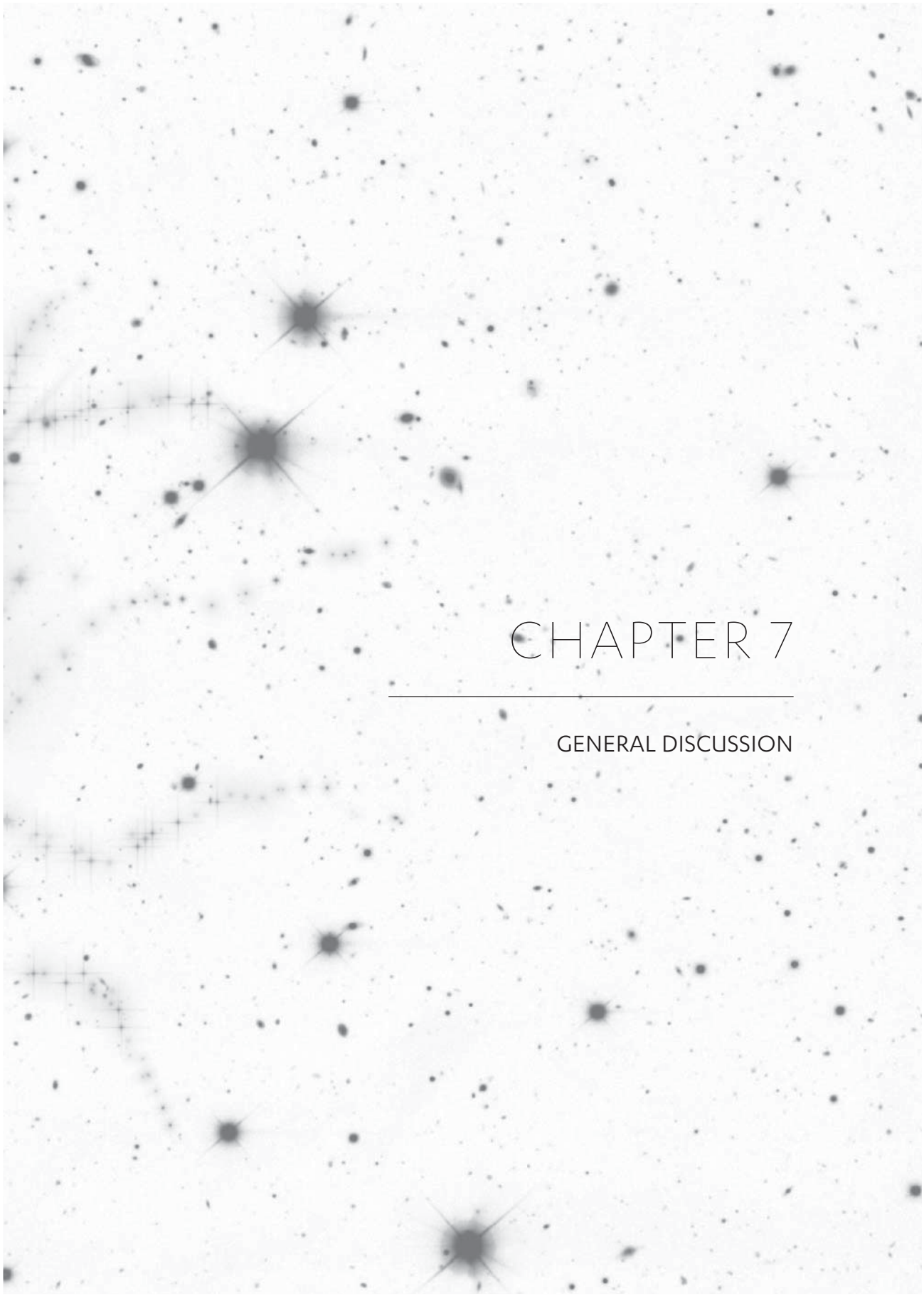
Doc2 deletion strongly reduced DCV release but did not abolish release. This suggests that other calcium sensors exist with a shared function in DCV release. These sensors could be synaptotagmin family members. Many synaptotagmins are expressed in brain with more than 12 isoforms being expressed in hippocampal neurons and several have high enough calcium sensitivity to be considered as candidates in DCV release (Hui et al., 2005). Syt-7, for instance, is expressed in hippocampal neurons and is enriched in terminals and has a higher calcium sensitivity than Syt-I. Its deletion does not affect SV release (Maximov et al., 2008) but strongly reduces DCV release from chromaffin cells (Schonn et al., 2008). Thus, Syt-7 is a strong candidate to function in neuronal DCV release. In fact, shRNA mediated silencing of Syt-7 in dopaminergic neurons results in decreased dopamine release from these cells (Mendez et al., 2011). Other synaptotagmins expressed in hippocampal neurons could also function in DCV release. In a screen using fusion proteins of the pH sensor pHluorin fused to the intraluminal domain of a number of synaptotagmin isoforms, Syt-9 fusion characteristics resembled those of DCV release (Dean et al., 2012). While the high-affinity calcium sensor Synaptotagmin 4, which co-localizes with BDNF-containing DCVs, is not a likely candidate as deletion increases DCV release (Dean et al., 2012). Future work will focus on the role of the different synaptotagmins in DCV release.

Alternatively, neuronal DCV release may be triggered in the absence of synaptotagmins. The binary Doc2/Munc13 complex might be sufficient to trigger calcium-dependent DCV release. The calcium dependent recruitment of this complex to the membrane may provide efficient release sites that by interacting with a SNARE/SM protein complex drive efficient vesicle fusion. This hypothesis can be tested in Munc13-1/Doc2ab triple null mutant neurons, where calcium-dependent DCV release should be completely abolished if no alternative calcium sensing pathways exist.

Our stimulation protocol resulted in robust translocation of Doc2 to the plasma membrane. Doc2 is not attached to the plasma membrane under resting conditions, but moves back and forth to the membrane upon repeated stimulation. DCV release has been shown to benefit from repeated bursts of stimulation (Bartfai et al., 1988; Hartmann et al., 2001; Iverfeldt et al., 1989), which suggests that this dynamic behavior of Doc2 might be associated with an underlying mechanism. Doc2 interacts with the dynein light chain tctex-1, which is involved in vesicular trafficking between membrane organelles (Nagano et al., 1998). Although we did not test this directly, this interaction might be important for DCV recruitment to release sites. Previously, we have shown that axonal transport of DCVs temporarily arrests upon calcium entry (de Wit et al., 2006). Hence, it is conceivable that during calcium influx, DCVs dissociate from the

microtubule network. These DCVs could then diffuse to the plasma membrane where they encounter the Doc2/Munc13 priming machinery promoting their fusion with the membrane. In Drosophila NMJ, specifically retrograde moving vesicles are recruited to release sites (Wong et al., 2012). Dynein is a retrograde transport molecule in axons, and a similar mechanism could therefore apply to the transport of neuronal DCVs. In this model, Doc2 might function at the initial step where DCVs leave the microtubule track by forcing DCVs propelled by dynein to detach from the tubulin network via calcium-dependent binding to tctex-1. Future experiments that focus on the dynamics of DCVs prior to release in Doc2 DKO neurons will test whether Doc2 may function as a link between long-range trafficking and release of DCVs.





CHAPTER 7

GENERAL DISCUSSION

The main aim of this thesis is to provide mechanistic insight in neuronal DCV release and to characterize the molecules that drive DCV fusion in neurons. In the introduction we pointed out that neuropeptide release from DCVs modulates many aspects of neuronal function: trophic factors like BDNF regulate neuronal connectivity and modulate presynaptic output (Dean et al., 2009; Kuczewski et al., 2008; Kuczewski et al., 2009; Kwinter et al., 2009; Lessmann and Brigadski, 2009), proteolytic enzymes cleave extracellular proteins, which allows for structural plasticity (Frischknecht et al., 2008; Gualandris et al., 1996; Kwinter et al., 2009; Lochner et al., 2006; Silverman et al., 2005), guidance cues navigate axons to their appropriate target (Cho et al., 2012; de Wit et al., 2006b; de Wit et al., 2009; Zylbersztejn et al., 2012) and trans-synaptic signaling molecules like SynCAM1 dynamically control synapse number (Robbins et al., 2010). In addition, specialized DCVs or PTVs (Piccolo-Bassoon transport vesicles) transport pre-assembled release machinery for synaptic transmission during synapse development (Shapira et al., 2003). Hence, it stands out that DCV signaling is a central factor in tuning brain function.

In this thesis, we analyzed the temporal and spatial aspects of neuronal DCV release and investigated several protein families with distinct functions in the release of synaptic vesicles for their role in DCV release. We found that DCV release is dependent on the SNARE fusion machinery, but that different isoforms of VAMP and SNAP25/23 are involved during different stages of development. Surprisingly, the SM protein Munc18-1 is not essential for DCV release in immature neurons, nor can it boost release when overexpressed in mature neurons. Synaptic localization of DCV release is regulated by the synaptic protein Munc13-1, while CAPS regulates DCV release efficiency independent of spatial location. A global and rhythmic calcium rise activating high affinity calcium sensors like Doc2b appears to be the most efficient way to trigger DCV release. Overall, the fact that neuronal DCV release behaves differently from and uses different release machinery components then DCV release in chromaffin cells and SV release, shows that the neuronal DCV release machinery has evolved together with the complexity of the neuron.

In *Chapter 2* we investigated the location of DCV release. We found that DCVs are released from synaptic and extra-synaptic sites. DCV release occurred preferentially at synapses and synaptic release required less robust stimulation than extra-synaptic release. The synaptically localized priming protein Munc13-1 regulated this synaptic specificity and increased release probability at the synapse. In addition, Munc13-1 is sufficient to make extra-synaptic release as efficient as synaptic release.

In *Chapter 3* we investigated how CAPS proteins regulate DCV release. CAPS and Munc13-1 share an important priming domain, and CAPS deletion resulted in a similar reduction of DCV release efficiency as observed in *munc13-1/2* null mutant neurons. In contrast to Munc13 deletion however, CAPS deletion did not affect the location of release.

Chapter 4 focused on the involvement of the SM protein Munc18-1 in DCV release. We obtained unexpected results that stand in contrast to the regulation of DCV release from adrenal chromaffin cells, as deletion of Munc18-1 in immature neurons did not affect DCV release. Furthermore, Munc18-1 overexpression in mature neurons failed to increase DCV release efficiency, again different from DCV release in chromaffin cells. This suggests that both cell types use different release machineries to drive DCV release during different stages of development.

Chapter 5 focused on the proteins that assemble into the SNARE complex: VAMP2, syntaxin-1 and SNAP25. We showed that a Botulinum toxin C-sensitive syntaxin (syntaxin-1, -2 or -3) is essential for DCV release, but that other VAMP isoforms than the canonical VAMP2 isoform are involved. Especially

during development release was insensitive to Tetanus toxin suggesting a role for TI-VAMP (VAMP7) in DCV release at this developmental stage. Furthermore, *SNAP25* null mutant neurons showed almost normal DCV release, suggesting that other SNAP isoforms can replace SNAP25 in the SNARE complex that drives DCV release. In addition to the temporal changes in the use of different SNARE complexes, the different sub cellular locations of SNARE protein isoforms, imply that different SNARE complexes could be responsible for fusion at different target membranes.

In *Chapter 6* we investigated which Ca^{2+}-sensors regulate DCV secretion. Deletion of the low-affinity calcium sensor synaptotagmin-I did not affect DCV release. But, deletion of the high-affinity calcium sensing protein Doc2 strongly reduced DCV release, suggesting that DCV release is triggered by a rise in global calcium rather than the local high concentrations of calcium that trigger synchronous synaptic vesicle release. Like deletion of Munc13-1, deletion of Doc2 affected the synaptic preference of DCV release. This suggests that a complex of both interacting proteins is involved in synaptic targeting of DCV release.

THE MINIMAL REQUIREMENTS OF DCV RELEASE SITES AND THEIR LOCATION

In *Chapter 2* we found that, although DCVs are not enriched at synapses, DCVs fuse preferentially at synaptic sites. This higher release probability at synapses is dependent on the active zone specific protein Munc13-1, which is also an important priming factor for synaptic vesicle release (Varoqueaux et al., 2002). Importantly, ectopic expression of Munc13-1 conferred synaptic like properties to extra-synaptic sites. Hence, efficient release sites require the presence of a single priming protein in addition to functional SNARE/Munc18 complexes, which are not restricted to synaptic terminals. Munc13 may increase extra-synaptic release probability by activating extra-synaptic SNARE/Munc18 complexes. The α-helical and autonomously folded MUN-domain in Munc13 may play an important role by accelerating the transition of syntaxin-1/Munc18 complexes to fusion competent SNARE complexes (Basu et al., 2005; Ma et al., 2011; Rizo and Sudhof, 2002). Whether Munc13-1 is unique in this respect is unknown. Deletion of the synaptically enriched protein CAPS, containing the same MUN-domain as Munc13-1, did not specifically decrease release at synaptic sites (*Chapter 3*). This suggests that the mere presence of a MUN-domain is not sufficient to confer synapse specificity of DCV release. We did however not test if ectopic overexpression of CAPS results in enhanced extra-synaptic DCV release. We found that deletion of Doc2 also abolished synaptic preference. Doc2 and Munc13 interact in a calcium dependent manner (Orita et al., 1997) and this interaction has been implicated in vesicle release in several secretory systems (Higashio et al., 2008; Mochida et al., 1998). We now also identify this binary complex as a complex that specifies the spatial location of DCV release. In this model Munc13 recruits Doc2 to presynaptic terminals to locally increase membrane fusogeneity.

Why are synaptic terminals preferred sites of DCV release? Many of the neuropeptides released from DCVs function to modulate neurotransmitter release, either from the post-synaptic site signaling to the presynaptic site or from axo-axonic synapses directly controlling the output of the targeted synaptic terminal (Lessmann et al., 2003; Luikart et al., 2008). In addition, presynaptic DCV release also tunes postsynaptic responses (Luikart et al, 2008). For these short-range local actions it is important that release of a single or a few DCVs results in a sufficiently high neuropeptide concentration to activate receptors on neighboring cells or

underlying terminals. Hence, co-release of DCVs and SVs at single synapses is an attractive mechanism to achieve such high local concentrations.

DCV RELEASE SITES IN VIVO

DCV release from non-synaptic sites along the neurites occurs only after prolonged stimulation. In fact, the stimulation pattern required to induce substantial extra-synaptic DCV fusion, more than 4x 50 action potentials at 50 Hz, seems too strong to occur often in vivo. Although we cannot exclude that effective stimuli for non-synaptic release are different in vivo, our findings suggest that given the activity patterns occurring in vivo, DCV release may be largely or almost exclusively synaptic in central neurons. This conclusion is consistent with the fact that DCV fusion delivers, in addition to classical neuropeptides, many substances that provide spatially restricted signals, such as guidance cues, trans-synaptic signaling molecules or, in developing neurons, pre-assembled active zones (Tran et al., 2009; Zhai et al., 2001).

THE MOLECULAR IDENTITY OF DCV CORE RELEASE MACHINERY

In *Chapter 5* we found that DCV fusion uses different SNARE proteins than SV fusion. In addition, SNARE protein usage depended on the developmental stage of the neurons. It is conceivable that these different SNARE proteins reflect the use of different SNARE complexes during development which is probably also depending on the sub-cellular localization.

In mature neurons, deletion of SNAP25 does not affect the number of DCV release events upon stimulation. However, DCV release requires more prolonged stimulation. Hence, the SNAP isoform that drives release in these neurons is less efficient than SNAP25. SNAP23 is the major SNAP isoform that could function in SNAP25 null neurons. This isoform is mainly expressed in dendrites (Suh et al., 2010), which may suggest that in wildtype neurons dendritic release is mainly controlled by SNAP23 containing SNARE complexes and axonal release by SNAP25 containing SNARE complexes. Although we did not directly test this hypothesis, our data is in line with previous observations (Matsuda et al., 2009) that revealed kinetic differences between axonal and dendritic release. Future research will be directed towards identifying axonal and dendritic DCV release machinery. Of interest is that dendritic release of AMPA receptor-containing recycling compartments is mediated by Syntaxin-4 containing SNARE complexes (Kennedy et al., 2010). Syntaxin-4 is insensitive to Botulinum toxin C (Schiavo et al., 1995). Hence, the complete arrest of DCV fusion that we observed upon treatment with BoNTC (*Chapter 5*) suggests that indeed different SNARE complexes drive release of different exocytic vesicles.

HOW IS DCV RELEASE TRIGGERED? THE Ca^{2+}-SENSORS THAT REGULATE DCV SECRETION

Unlike SVs that can be refilled with neurotransmitters locally at the synaptic terminal, neuropeptides are synthesized at the endoplasmatic reticulum and loaded into DCVs at the Golgi apparatus. DCVs then travel from the Golgi through the neurites along microtubule

tracks from which they have to be released before fusing with the target membrane. DCVs are highly mobile, which provides the neuron with many trafficking vesicles that can be recruited to release sites upon demand. But how are these vesicles recruited? We have found previously (de Wit et al., 2006b) that DCVs stall on the microtubule track upon stimulation, suggesting that calcium levels influence the trafficking of DCVs. One attractive hypothesis holds that the stalling represents the temporal dislodging of the DCV from the tubulin track allowing the DCV to diffuse to the plasma membrane where it may be captured by receptive SNARE complexes. How DCVs sense the global rise in calcium and how this translates to stalling and the occasional fusion of DCVs upon stimulation is unknown. However, in *Chapter 6* we provide evidence that a high affinity calcium sensor, Doc2, may be an important protein to translate global rises in calcium into increased DCV release probability. In addition we show that the canonical low affinity synaptogmin-1 does not function in DCV release, which is in line with the notion that DCVs fuse from an undocked mobile pool and not from a pre-docked (synaptotagmin-1 sensitive) pool. DCV release is not fully abolished in the absence of Doc2, which indicates that other, most likely high affinity calcium sensors, may operate in DCV release.

DCV RELEASE DURING DEVELOPMENT

Our data in *Chapter 4* on DCV release in immature and mature neurons shows that release efficiency is quite similar between the two developmental stages. Immature neurons have fewer vesicles, but release a similar percentage of their vesicle pool upon stimulation. However, release in immature neurons is less strictly timed to the stimulus as in mature neurons and release tends to continue beyond the stimulation period when calcium levels are low. This may be caused by differences in the release machinery in developing neurons (see below) or in calcium-dependent mechanisms such as protein phosphorylation that increase release probability for longer periods in developing neurons.

We investigated the role of SNAP25, Munc18-1 and VAMP in immature and mature neurons and found that the requirement of these proteins depends on the developmental stage of the neuron. Release in immature neurons can take place in the absence of SNAP25, Munc18-1 and all cleavable VAMP isoforms. This suggests that other isoforms of these proteins can form functional DCV release machinery in immature neurons. An uncleavable isoform of VAMP, TI-VAMP (VAMP7), is expressed during neuronal development, where it regulates outgrowth of axons and dendrites (Martinez-Arca et al., 2001), forming a complex with syntaxin-1 and SNAP25 at the plasma membrane (Martinez-Arca et al., 2000). In epithelial cells, TI-VAMP forms a complex with syntaxin3 and SNAP23 at the apical membrane regulating apical transport (Coco et al., 1999). One or both of these SNARE complexes could also play a role in DCV release in immature neurons.

Our data suggest that neuronal DCV release is driven by at least two different DCV release complexes that function during distinct periods in development (Figure 1). During early development a complex consisting of SNAP23, VAMP7 and syntaxin-1, -2 or -3 regulates vesicle release from extending neurites. In mature neurons, the canonical SV release complex (syntaxin-1, SNAP25 and VAMP2) controls fast synaptic DCV release. Most likely, the synaptic release we observe in our paradigm is from axonal, presynaptic origin, in line with the presynaptic expression of SNAP25 and VAMP2. In contrast, SNAP23 expression in mature neurons is mainly postsynaptic and the SNAP23 containing SNARE complex that functioned during early

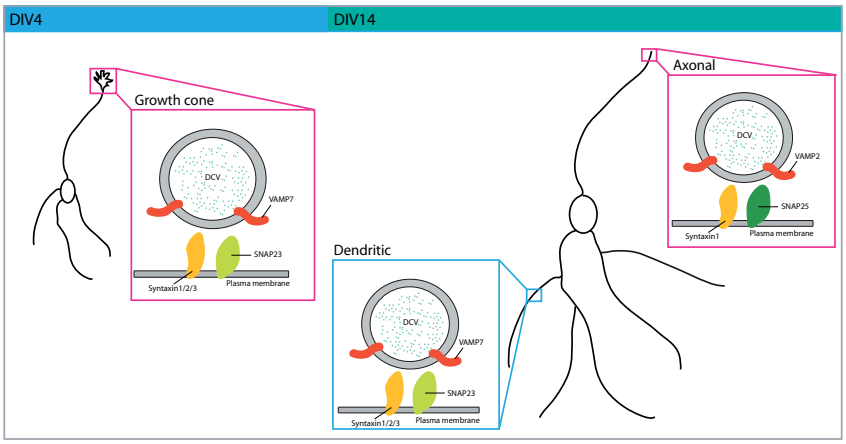


Fig 1 Model 'DCV SNARE complexes during development. During early development in neurons in culture until approximately DIV4, neurites are not yet segregated into axons and dendrites on the molecular level. Our data shows that young neurons do not use SNAP25 and VAMP-1, -2 or -3 for DCV fusion at DIV4, but might instead use a complex of VAMP7, SNAP23 and syntaxin-1, -2 or -3. When the neuron develops further, protein segregation into axons and dendrites creates two different DCV SNARE complexes: The canonical axonal SV SNARE complex regulates axonal DCV release whereas a complex consisting of VAMP7, SNAP23 and Syntaxin-1, -2 or -3 regulates dendritic DCV release.

development now becomes dendritic postsynaptic release machinery. In line, VAMP7 also localizes at somato-dendritic sites after the onset of synaptogenesis in hippocampal neurons (Coco et al., 1999). Hence, DCVs use evolutionary old SNARE machinery to drive release in developing neurons and from dendritic compartments. Fast synaptic release uses similar and evolutionary younger SNARE machinery that drives synaptic vesicle release.

Immature *munc18-1* null mutant neurons showed normal DCV release. As vesicle fusion requires the presence of an S/M protein, this suggests that other SM proteins are involved in the regulation of DCV release during development. Munc18-2 and Munc18-3 are both expressed in brain, albeit at relatively low levels, and could function in DCV release during development. Munc18-3 functions in the release of GLUT4 vesicles from beta-cells together with the SNARE complex consisting of SNAP23, VAMP2 and Syntaxin4 (Tamori et al., 1998; Thurmond et al., 1998). Munc18-2 controls the association of SNAP23 and cellubrevin (VAMP1) with syntaxin-3, regulating apical membrane transport in epithelial cells (Riento et al., 1998). Munc18-2 also interacts with syntaxin-11 in lymphocytic cells, regulating granule exocytosis (zur Stadt et al., 2009).

Thus, during development different Munc18 isoforms work together with different SNARE complexes that may drive DCV release. Preliminary results from our lab show that deletion of Munc18-1 in mature neurons (using inducible *munc18* null mutant mice) does result in complete arrest of DCV release. Hence, during development neurons switch Munc18 isoforms (and SNARE complexes) to drive DCV release. Currently, we are testing which Munc18 isoform is used during development.

DOCKING OF VESICLES

We found striking differences in DCV release from neurons and chromaffin cells in terms of the proteins needed for vesicle release. Our analysis of vesicle location and dynamics revealed one of the biggest differences between DCVs in chromaffin cells and DCVs in neurons. In contrast to DCVs in chromaffin cells, DCVs in neurons are very mobile, do not accumulate at release sites and are rarely pre-docked at their target membrane. Hence, neuronal DCVs are recruited to the target membrane during activity and thus dock and fuse from an undocked, mobile state during stimulation. This is in line with our observation that the major docking proteins in chromaffin cells, Munc18-1, SNAP25 and synaptotagmin-I do not play a major role in neuronal DCV release. However, we couldn't fully investigate the need for munc18-1 and SNAP25 in mature neurons due to the early cell death in these cultures. Impaired docking of SVs or DCVs in chromaffin cells, results in severely reduced release of these vesicles (de Wit, 2010; de Wit et al., 2006a; Voets et al., 2001). How can this important step be of less importance in neuronal DCV release? One explanation may come from differences in timing of release. SV release in neurons is a fast and accurately-timed process of information transfer and acute adrenaline release from DCVs in chromaffin cells is vital for an immediate flight-fight response of the organism. In contrast, neuropeptides released from neuronal DCVs modulate neurotransmitter release, which allows for a somewhat slower modulatory response from recruited DCVs. The docking of vesicles might therefore reflect the need to fuse multiple vesicles at the same time, which is not necessary for DCV release. A mobile non pre-docked DCV population in neurons assures that release sites can be (re-) supplied with DCVs according to demand.

MODEL OF DCV RELEASE

Based on our findings in this thesis we propose a model for neuronal DCV release. First, calcium influx upon stimulation arrests DCV transport along microtubules (de Wit et al., 2006b). This may lead to dissociation from the tubulin track or motor proteins allowing DCVs to freely diffuse and bind receptive tSNARE-proteins (acceptor complexes) at the target membrane to dock and fuse. High affinity calcium sensors like Doc2 may convey the calcium signal to the transported vesicles but may also operate after DCV dislodgment from the tubulin track (Figure 2). DCV docking might be a more transient state for DCVs, avoiding the need for specific docking factors that function in DCV release from chromaffin cells. Munc13 and CAPS proteins may promote the formation of receptive tSNARE complexes (Basu et al., 2005; Hammarlund et al., 2008; Khodthong et al., 2011; Ma et al., 2011; Weninger et al., 2008). These tSNARE complexes are likely to differ in their composition depending on their sub cellular location and developmental stage. In wild type neurons, Munc13 distribution is restricted to active zones and DCV fusion is therefore mostly synaptic and many DCVs may re-attach to the tubulin track when intra-cellular calcium levels drop. Intense prolonged stimulation may trigger additional mechanisms independent of Munc13 and CAPS to promote acceptor complex availability, which renders DCV-fusion Munc13/CAPS-independent during intense activity.

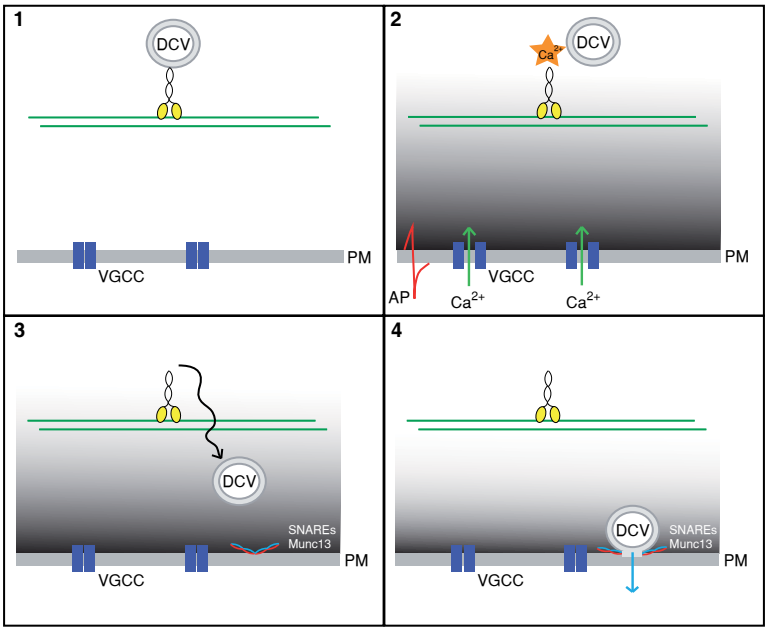
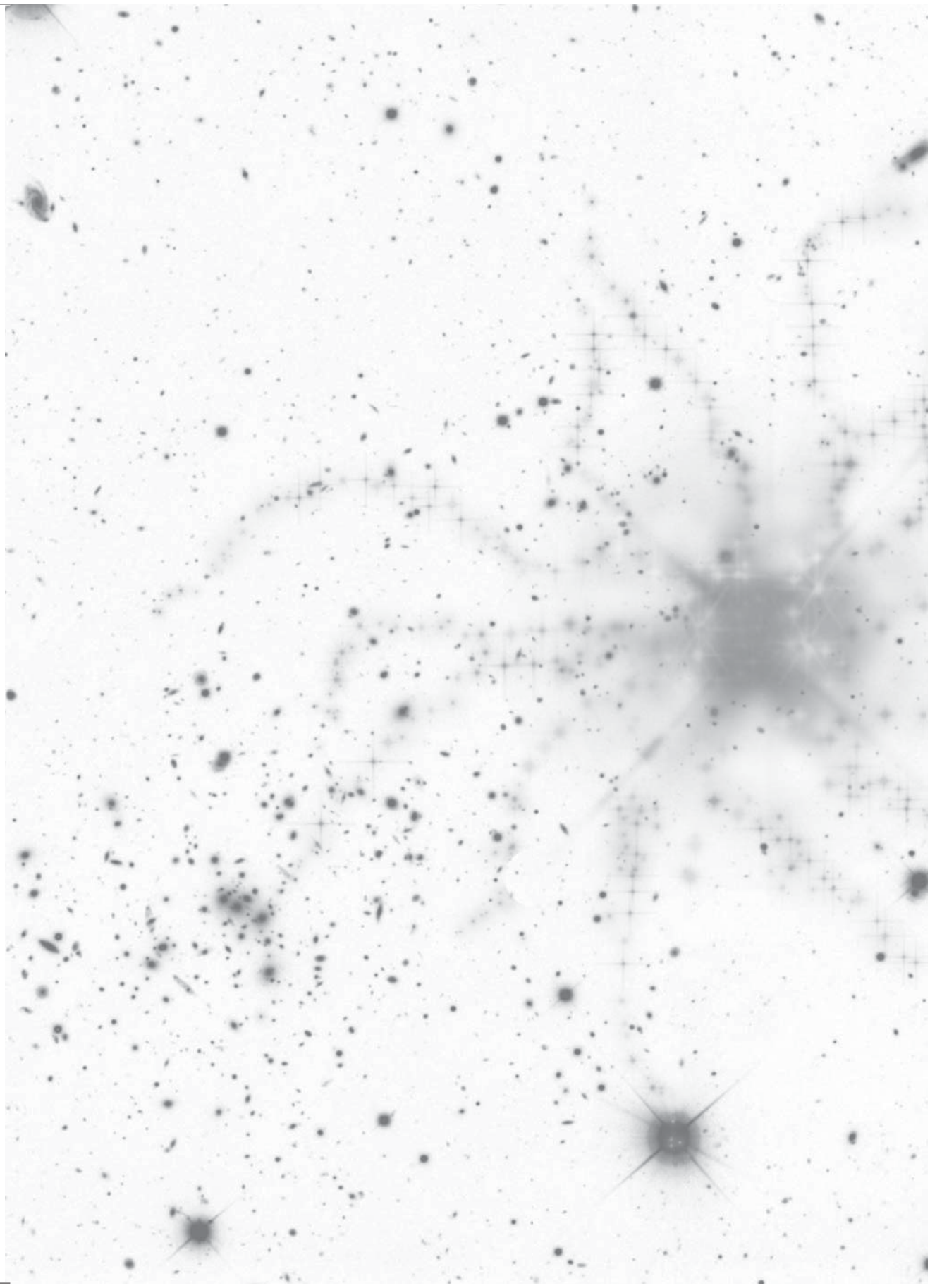


Fig 2 Model 'DCV trafficking to release. (1) During resting state, DCV traffic along microtubules attached to motor proteins (e.g. KIF1A). (2) DCVs stall upon calcium influx through voltage gated calcium channels (VGCC) triggered by an action potential (AP). Calcium levels diffuse through the neurite and release the DCV from the motor protein. (3) The free DCV diffuses towards the plasma membrane (PM). (4) Once it reaches the plasma membrane it binds to the SNARE machinery and the synaptic protein Munc13-1 and fuses with the plasma membrane releasing its contents in the extra-cellular space.

CONCLUSION AND FUTURE DIRECTIONS

It is beyond doubt that DCV signaling is a central factor in brain function. In this thesis we studied the molecules that control the calcium-dependent release of DCVs. We found that neuronal DCV release mechanisms differ from those used for DCV release in chromaffin cells and synaptic vesicle release from neurons. In addition, we identified the minimal release machinery for neuronal DCV release (SNARE/SM complex and Munc13), show that CAPS proteins are major DCV priming factors and that the high affinity calcium sensor Doc2 is the major calcium sensor for DCV release from neurons.

One major question that remains is how neurons distinguish between DCVs with different cargo. We studied the behavior of the average DCV as our transfection method labels all DCV in a neuron. Future experiments will be focused on the behavior of specific subtypes of DCVs. This means that we need to label endogenous cargo and set out to identify the specific markers of different subtypes of DCVs. Another important issue to address is our current lack of understanding of the mechanism that recruits DCV to release sites. Especially the step from tubulin detachment leading to vesicle fusion is still not understood. Current experiments in the lab using DCV cargo with two fluorescent labels that allows the visualization of vesicle transport and fusion are tailored to increase our knowledge of the steps just prior to vesicle fusion.



CHAPTER 8

MATERIALS AND METHODS

PLASMIDS

SemapHluorin was generated by replacing EGFP in Sema3A-EGFP (De Wit et al., 2005) with the super-ecliptic pHluorin (SpH) coding sequence (de Wit et al., 2009). Neuropeptide Y (NPY)-SpH was generated by replacing Venus (Nagai et al., 2002) with SpH (de Wit et al., 2009). Synapsin-mCherry was a kind gift of Dr. A. Jeromin (Allen Brain Institute, Seattle, USA). pBont/cIRESpvECFPcDNA3 was used to express BoNT-C (de Wit et al, 2006) and pTentIRESpvmRFP-N1 to express Tetanus toxin. Munc18-1-cherry was published before (Toonen et al, 2006) as well as Doc2b-RFP (Groffen et al, 2006).

LABORATORY ANIMALS

Munc13-1/2 double knockout (M13DKO) mice have been described before (Varoqueaux et al., 2002). E18 embryos were obtained by caesarean section of pregnant females from timed matings of Munc13-2 homozygous and Munc13-1 heterozygous mice. *Munc18-1, Synaptotagmin-I* and *SNAP25* null mutant mice were generated as described previously (Geppert et al., 1994; Verhage et al., 2000; Washbourne et al., 2002) and E18 embryos were obtained from timed matings of heterozygous mice. CAPS-1/2 null mutant mice were generated as described before (Jockusch et al., 2007) and E18 embryos with either CAPS-1, CAPS-2 or CAPS-1/2 deletion were obtained from timed matings of CAPS-2 homozygous and CAPS-1 heterozygous mice. Doc2 null mutant mice were generated as described previously (Groffen et al., 2010) and heterozygous E18 embryos were obtained by timed matings of Doc2 heterozygous mice.

Munc13-1/2 DKO, Munc18-1 KO, SNAP25 KO and CAPS1/2 DKO embryos are all still born, but for all genotypes the correct genotype of the embryos was confirmed by genotyping the embryonic tail. Newborn P0-P1 pups from pregnant female Wistar rats (Harlan or Charles River) were used for glia preparations. Animals were housed and bred according to institutional, Dutch and U.S. governmental guidelines.

GLIA PREPARATION

Forebrains of newborn (P0-P1) rat pups were dissected in ice-cold Hanks Buffered Salt Solution (HBSS, Sigma) buffered with 7 mM HEPES (Invitrogen). After removal of the hippocampus, hemispheres were incubated in 20-25 U/ml papain enzyme solution for 60 min at 37°C. After washing, cells were triturated using a firepolished Pasteur pipette. Cells from one animal were plated in pre-warmed DMEM medium (Invitrogen) supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids (NEAA) and 1% pen/strep (all from Gibco) in a T175 flask. Medium was refreshed the next day. Cells were allowed to grow for 1 week at 37°C/ 5% CO2. After PN02, cells were trypsinized and frozen and stored in liquid nitrogen. Cells were allowed to recover and divide for 1 week after thawing before further use.

PRIMARY NEURONAL CELL CULTURE

Dissociated hippocampal neurons were prepared from embryonic day 18 mice as described (de Wit et al., 2009). Hippocampi were dissected in Hanks Buffered Salts Solution supplemented

with HEPES (HBSS, Sigma) and digested with 0.25% trypsin (Invitrogen) for 20 min. at 37˚C. Hippocampi were washed and triturated with fire-polished Pasteur pipettes, counted and plated in Neurobasal medium (Invitrogen) supplemented with 2% B-27 (Invitrogen), 1.8% HEPES, 1% glutamax (Invitrogen) and 1% Pen-Strep (Invitrogen). High-density cultures (25k neurons/well) were seeded on pre-grown cultures of rat glia cells (PN02, 37,5k cells/well) on PDL (0.1 mg/ml, Sigma) and collagen coated (0.2 mg/ml, BD biosciences) 18mm glass coverslips in 12-well plates.

CALCIUM PHOSPHATE TRANSFECTION

Neuronal cultures were transfected at DIV2 or DIV10 with calcium phosphate with DCV-pHluorin together with synapsin-mCherry (to identify synapses) and ECFP (as neuronal morphology marker). Neurons were imaged at DIV4 for developmental studies and DIV14-DIV15 for studying the adult nervous system. Neurons were transfected according to the calcium-phosphate transfection method described previously (Kohrmann et al., 1999).

LIVE CELL IMAGING

All experiments performed were blinded before imaging and only revealed after analysis was completed. Coverslips were placed in an imaging chamber and perfused with Tyrodes (2 mM CaCl2, 2.5 mM KCl, 119 mM NaCl, 2 mM MgCl2, 20 mM glucose and 25 mM HEPES, pH 7.4) and imaged on an Axio Observer Z1 microscope (Zeiss) equipped with a Coolsnap HQ camera (Photometrics) and a Polychrome IV illumination unit (TILL Photonics). Images were acquired at 2 Hz with Metamorph 6.2 software (Universal Imaging) using a 40x objective (NA 1.3). Intracellular pH was neutralized with normal Tyrode's solution containing 50 mM NH_4Cl, which replaced NaCl on an equimolar basis in the solution. A barrel pipette was used to apply NH_4^+ solution to the cells. Electrical field stimulation by parallel platinum electrodes was applied by a Master 8 system (AMPI) and a stimulus generator (A385RC, World Precision Instruments) delivering 30 mA, 1 ms pulses. The stimulus used was 16 bursts of 50 action potentials at 50 Hz with 0.5 s interval. All imaging experiments were performed at room temperature (21-24 °C) in the presence of 50 µM APV (Tocris) and 10 µM DNQX (Tocris) to block glutamatergic transmission. The imaging protocol consisted of a 30 s time-lapse, applying NH_4^+ Tyrodes solution after 10 s for 10 s. Then a 5-minute recovery period followed, in which spontaneous release was assessed during the last 60 s of this period. After this, a 100 s time-lapse with electrical field stimulation was recorded. Field stimulation started after 3 s and lasted 24 s. Spontaneous events after the stimulation were counted during the last 70 sec. Furthermore, ECFP and synapsin-mCherry images were acquired before and after the recording for further analysis. Neurons in which ECFP or synapsin-mCherry masks before and after stimulation shifted more than 1 pixel (0.165 µm) were discarded from analysis.

IMAGE ANALYSIS

Stacks from time-lapse recordings acquired with 0.5 s intervals were used to analyze DCV release. A 4x4 pixel region (0.6 x 0.6 µm) of interest was centered on each event, and the average intensity of the fluorescence was measured with Metamorph software (Universal

Imaging). Fluorescence traces were expressed as the fluorescence change (ΔF) compared to the initial fluorescence (F_0), obtained by averaging the first 4 frames of the time-lapse movie. Onset of exocytosis was defined as the first frame with an increase of fluorescence of two standard deviations above F_0. DCV release rates were measured from linear fits of the cumulative plots. Co-localization with synapsin was measured by overlaying both images in Metamorph. A cargo-pHl release event or punctum was scored as synaptic when the fluorescence center of such a release event/punctum was within 200nm (±1 pixel, the approximate minimal point spread function of our system) of the synapsin-mCherry fluorescence centroid. Extra-synaptic events were all events that did not meet this criterium. We only measured release events from neurites and excluded somatic release events. Somatic release events cannot be reliably measured using wide-field fluorescence microcopy due to the bright fluorescence from vesicles in/near the Golgi apparatus in which the intraluminal pH is not yet acidic. The total number of vesicles was automatically analyzed from the NH_4^+ application time lapse using SynD software (Schmitz et al., 2011). In Chapter 2, the localization and dynamics of DCVs was determined by analyzing Cargo-pHluorin labeled vesicles during NH_4^+ application. Per neuron, four 50μm regions containing synapses were selected for linescan measurements. DCV fluorescence peaks 1 SD above average were scored for overlap with synapsin-mCherry fluorescence peaks at the first frame of a 10 s time window. During these 10 seconds, even the slowest moving DCVs in our system (0.29 μm/s, de Wit et al., 2006), would have traveled ± 3μm (or >18 pixels). Stable DCVs are therefore defined as DCVs that do not leave the synaptic area in 10 seconds.

Intensity measures of overexpressed proteins were analysed with Metamorph software (Universal images), measuring the average intensity of two random somatic regions (excluding the nucleus).

IMMUNOCYTOCHEMISTRY

After imaging, cells were fixed in 4% formaldehyde (Electron Microscopies Sciences) in PBS, pH7.4 for 20 min at room temperature (RT). For staining with secretogranin II antibody a different fixation procedure was followed, adding ice-cold methanol for 10 min. after 8 min. fixation in 4% FA. Cells were washed in PBS and first permeabilized for 5 min in PBS containing 0.5% Triton X-100 (Sigma-Aldrich) then incubated for 30 min with PBS (Gibco) containing 2% normal goat serum and 0.1% Triton X-100. Incubations with primary and secondary antibodies were done for 1 hour at RT. Primary antibodies used were; polyclonal MAP2 (Abcam), monoclonal VAMP2 (SySy) and polyclonal Munc13 (SySy), polyclonal chromogranin A, polyclonal secretogranin II (kind gift from P. Rosa, Institute of Neuroscience, Milan, Italy), polyclonal SNAP23 (SySy), monoclonal SNAP25 (Sternberger monoclonals), polyclonal Munc18-1. Alexa Fluor conjugated secondary antibodies were from Invitrogen. Coverslips were mounted in Mowiol and examined on a Zeiss LSM 510 confocal laser-scanning microscope with a 40x objective (NA 1.3).

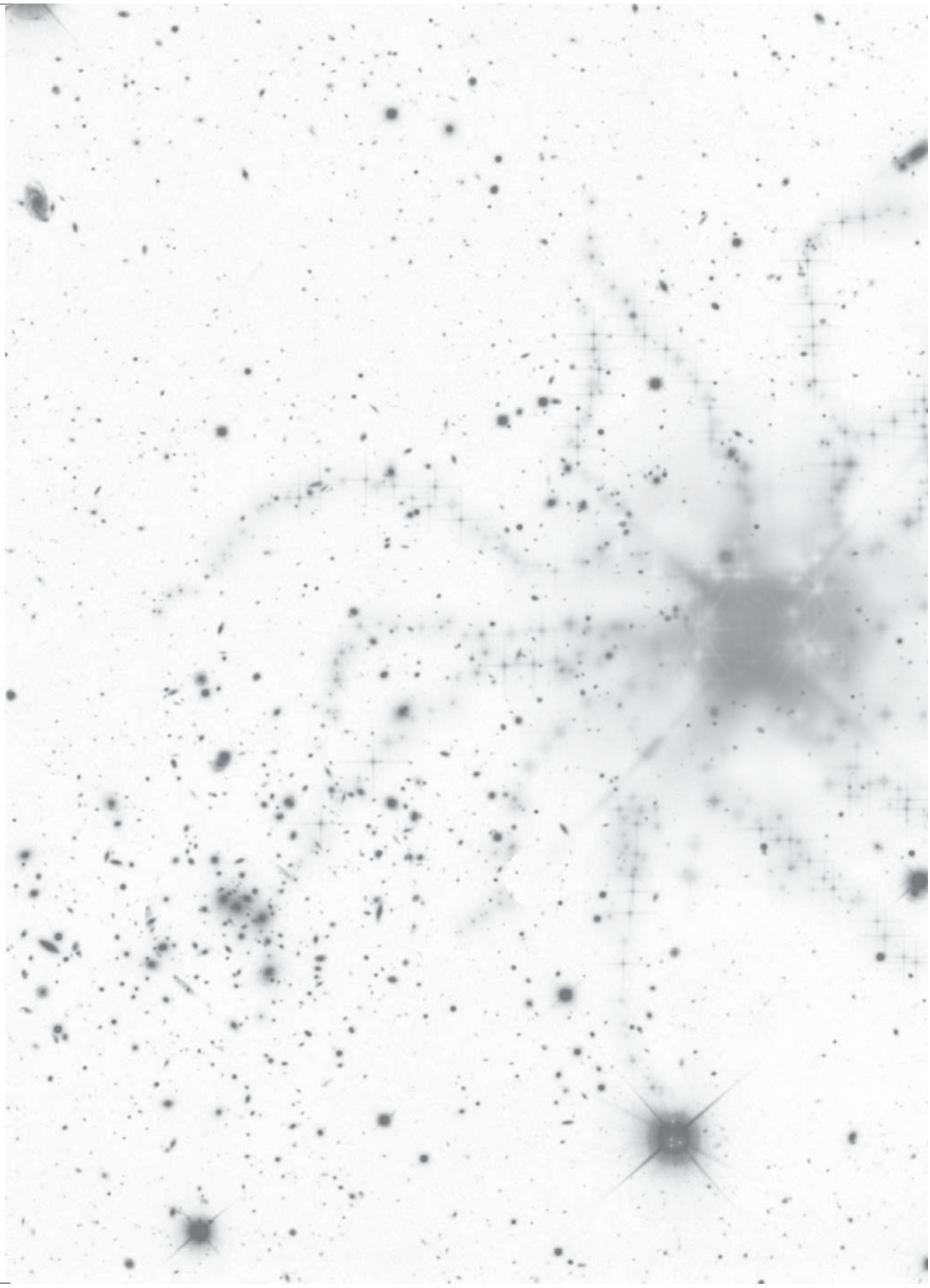
ELECTRON MICROSCOPY

Neurons were fixed at DIV14-16 for 1-2 hours at room temperature with 0.1 M cacodylate buffer/0.25mMCaCl2/0.5mM MgCl2 (pH 7.4) and processed as described (Meijer et al., 2012; Wierda et al., 2007). Briefly, cells were post-fixed for 2 hr at room temperature with 1% Osmium tetroxide/1% Potassium ferro-cyanide, washed and stained with 1% uranyl acetate for 40 min in

the dark. Following dehydration, cells were embedded in Epon. Cells of interest were selected using the light microscope, and mounted on pre-polymerized Epon blocks for thin sectioning. Ultrathin sections (~ 90 nm) were cut parallel to the cell monolayer, collected on single-slot, formvar-coated copper grids, and stained in uranyl acetate and lead citrate. Synapses with a recognizable pre- and postsynaptic density were randomly selected using a JEOL 1010 electron microscope and imaged at 100.000 x magnification using analysis software (Soft Imaging System, Gmbh, Germany). The observer was blinded for the genotype. DCV distribution was measured with Image J (National Institute of Health, USA). Docked DCVs were 0 nm from the vesicle membrane to the plasma membrane.

STATISTICS

Throughout this thesis, Student's t tests for unpaired data were used unless otherwise specified. If F-tests showed significantly different standard deviations, t tests were Welch corrected. Mann-Whitney tests were used to compare groups when one or both did not pass the normality test. To test more than 2 groups, Kruskal-Wallis one-way analysis of variance with post-hoc Dunn's multiple comparisons test was used. Kolmogorov-Smirnov test was used to test whether distributions were normally distributed and for testing frequency distributions. Data are plotted as average with SEM.



CHAPTER 9

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